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Multi-Level Regulation Of Argininosuccinate Synthase: Significance For Endothelial Nitric Oxide Production

Karen Davidowitz Corbin
University of South Florida

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Multi-Level Regulation Of Argininosuccinate Synthase:
Significance For Endothelial Nitric Oxide Production

by

Karen Davidowitz Corbin

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Molecular Medicine
College of Medicine
University of South Florida

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phosphorylation, post-translational modifications, heat shock protein 90, caveolin,
endothelial nitric oxide synthase, subcellular localization, protein interactions

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DEDICATION

First and foremost, I would like to dedicate this work to God. My prayer is that He can use the gifts He has given me as a scientist and educator to make positive contributions to our society. I would also like to dedicate this work to my family. My mom, Aurora, dad, Bob, and brother, Bobby, are my biggest fans and have supported me in all my endeavors. They have always inspired me to do my best and reach for the stars. My husband, best friend and chef- Stephen- has been a huge support during these years of sacrifice. Despite all the craziness of graduate school, he still chose to marry me. He has earned this degree just as much as I have. All my family- from The Davidowitz side and The Rodriguez side- have contributed to my life through their love, support and guidance. Finally, my newest family- The Corbin’s- have been such a wonderful addition to my life and have been rooting for me and supporting me from the day I met them. I do want to especially dedicate this work to my brave and precious nephew Ethan Corbin. He was diagnosed with neuroblastoma at six months of age and has fought like a brave little soldier to beat that disease. He is an inspiration and a survivor. If anyone ever wonders why someone would dedicate their life to science and healthcare, it is for little angels like him. Without the advances in medicine that have been made possible by scientific research, the gift of his life and love might have ceased to exist.
ACKNOWLEDGEMENTS

The scientific journey through graduate school is one full of many experiences. Some are expected and some are unexpected. Through the blood, sweat and tears, it takes an army of people to teach you the lessons that you never knew you would need to learn. Graduate school is a time where you see the significance of ethics and learn the most valuable skill in science— independent thinking. I certainly could not have made it through without the investment that many people made in me.

Dr. Eichler is a fantastic mentor. From the day I walked in his office unexpectedly and asked him if I could join his lab, he has treated me like a colleague and not a student. He always valued my opinions (even if he never actually asked for them). He allowed me the intellectual freedom to build my project from the ground up. Nothing can compare to the experience of figuring things out for yourself. He never pushed me because I push myself more than anyone, but he gently made suggestions that I eventually realized I should follow. One of the things I value about him the most is his attention to ethics. He never jumps to conclusions and has a true desire to make contributions to science that are based on truth. His concern is with improving the health of people, not pushing his own research agenda.
I am grateful to all the members of the Eichler Lab that became such a huge part of my graduate career. First, the Eichler and Solomonson labs have functioned as a single unit for many years. So, I consider Dr. Solomonson (Dr. Sol) a co-mentor. He was always available to help me with scientific questions. He was a great person to go to when things were all jumbled and I needed a clean-cut solution. He often came into the lab and said: “So, what have you discovered this week?” That level of confidence in me was extremely motivating. The Eichler lab literally took me in with open arms and did everything possible to make my transition into their lab a smooth one. I would like to thank Bonnie Goodwin, Brenda Flam and Laura Pendleton for their help and support when I first entered the lab. Their scientific contributions in our lab set an excellent foundation for the development of my projects. I especially want to thank Laura Pendleton. We went through a lot of transitions together- from department mergers to grant issues to moving into a new lab. Then, in between that, there was the science. Through it all, Laura was the one who kept me calm and helped me with both science and life issues. Finally, our newest lab member, Sandi Shriver, has been a huge, huge help in these last few months. Her willingness to help with all aspects of keeping our lab running has been more than I could have ever expected. She is an extremely bright person that works tirelessly. She will be successful in whatever path she chooses.

I would also like to recognize the contributions of my committee members: Denise Cooper, PhD, William Gower, PhD, Mark McLean, PhD, and Gene Ness, PhD. They were a very supportive committee that I could always count on to make it to meetings and give me good suggestions. They have cheered me on, pushed me and
guided me. I would also like to thank Dr. Chris Baylis from the University of Florida for so graciously agreeing to serve as the outside chair of my committee.

Thank you to all the collaborators that have contributed to this work: John Koomen, PhD, and Vicky Izumi at the Moffitt Proteomics Core, Marina Tran and Jasbinder Sanghera, PhD, at SignalChem and Wayne Guida, PhD, and Daniel Santiago at the USF Department of Chemistry.

I would like to thank a few colleagues and friends that have known me for many years. Cathy Levenson, PhD, and Jodee Dorsey, PhD, at Florida State University have become true life-long mentors. Anne Brezina and all the dietitians at the James A. Haley Veterans Hospital and the Tampa & Pinellas Dietetic Associations have been such wonderful friends and cheerleaders over the years. I am grateful to all my friends and colleagues at The Heart and Vascular Institute of Florida. The experiences I gained there will be an asset to my career forever and that was the birth place of my love of the science behind heart disease, diabetes and obesity.

Importantly, I definitely could not have made it without “a little help from my friends”. My best girlfriends, bridesmaids and sisters Paula Calabrese, Kelli Carr, Megan Orseck and Megan Sheiman have always been there for me. I am truly blessed for their impact in my life. I have many other wonderful friends that I am not mentioning individually but who are very important to me. Then there were many people who helped carry me through the days I thought I was not going to make it through graduate school.
It all began with Yira Bermudez and Shawna Shirley Gilman. We met in “Foundations in Biomedical Sciences”. We were so young, confused and naïve. Then we navigated through all the phases of graduate school together and helped each other through the good and bad times. Later in graduate school, Thomas Lendrihas joined our group. He definitely made graduate school bearable with his humor and unique point of view. For all the others who crossed my path - thanks! Each one of you touched my life and made a difference. One of my greatest hopes is that I can do for others what all the people in my life have done for me.
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“Troglitazone up-regulates vascular endothelial argininosuccinate synthase”

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<th>Description</th>
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<tr>
<td>Akt</td>
<td>RAC-alpha serine/threonine-protein kinase; Protein Kinase B</td>
</tr>
<tr>
<td>AL</td>
<td>Argininosuccinate Lyase</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine Monophosphate Activated Protein Kinase</td>
</tr>
<tr>
<td>ARP</td>
<td>Argininosuccinate Synthase Regulatory Protein</td>
</tr>
<tr>
<td>AS</td>
<td>Argininosuccinate Synthase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BAEC</td>
<td>Bovine Aortic Endothelial Cells</td>
</tr>
<tr>
<td>BH4</td>
<td>Tetrahydrobiopterin</td>
</tr>
<tr>
<td>CKII</td>
<td>Casein Kinase II</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial Nitric Oxide Synthase</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin Adenine Dinucleotide</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin Mononucleotide</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen Synthase Kinase 3 Beta</td>
</tr>
<tr>
<td>HSP90</td>
<td>Heat Shock Protein 90</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon Gamma</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible Nitric Oxide Synthase</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid Chromatography-Tandem Mass Spectrometry</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MDLA</td>
<td>$\alpha$-Methyl-DL-Aspartic Acid</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>Nickel Nitrilotriacetic Acid</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal Nitric Oxide Synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric Oxide Synthase</td>
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<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PKG</td>
<td>Protein Kinase G</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome Proliferator Activated Receptor</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-Translational Modifications</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozocin</td>
</tr>
<tr>
<td>TNF$\alpha$</td>
<td>Tumor Necrosis Factor Alpha</td>
</tr>
<tr>
<td>TZD</td>
<td>Thiazolidinedione</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular Smooth Muscle Cells</td>
</tr>
</tbody>
</table>
MULTI-LEVEL REGULATION OF ENDOTHELIAL ARGININOSUCCINATE SYNTHASE: SIGNIFICANCE FOR NITRIC OXIDE PRODUCTION

Karen Davidowitz Corbin, MS, RD

ABSTRACT

The citrulline-nitric oxide (NO) cycle, comprised of the enzymes argininosuccinate synthase (AS), argininosuccinate lyase (AL) and endothelial nitric oxide synthase (eNOS), is responsible for the regulated production of endothelial NO. Although most studies have focused on eNOS to uncover important regulatory mechanisms, we and others have determined that AS is an essential and regulated step in endothelial NO production. AS is rate limiting for endothelial NO production and is the primary source of arginine, the substrate for eNOS-mediated NO production, despite saturating intracellular levels of arginine and available arginine transport systems. AS is essential for endothelial cell viability and its expression is regulated coordinately with eNOS by TNFα and thiazolidinediones with concomitant effects on NO production. Given the importance of AS for endothelial health, we explored three independent regulatory mechanisms. In Chapter One, the functional consequences of altered AS expression due to overexpression, insulin, VEGF and ceramide were studied. We
demonstrated that overexpression of AS leads to enhanced NO production and that insulin, VEGF and ceramide coordinately regulate the expression of AS and eNOS. In Chapter Two, the first post-translational modifications of AS in the endothelium were characterized. We determined that AS is an endogenous phosphoprotein in the endothelium, described several levels of biological significance of AS phosphorylation, identified 7 sites of AS phosphorylation and began to uncover the direct impact of phosphorylation on AS function. Finally, in Chapter Three, endothelial AS subcellular localization was defined and important protein interactions were identified including caveolin-1 and HSP90. The work presented in this dissertation demonstrates that multiple mechanisms regulate the function of AS, often coordinately with eNOS, and have a direct impact on nitric oxide production. Our findings suggest that the global understanding of the citrulline-NO cycle as a metabolic unit will unravel new paradigms that will re-define our understanding of the regulation of vascular function by NO.
INTRODUCTION

Nitric Oxide: Historical Perspectives and Biochemistry

Nitric Oxide (abbreviation: NO; chemical formula: N≡O·) is a readily diffusing gas that can be poisonous in the environment yet powerful in biology [1, 2]. Due to its unpaired electron, NO is a free radical, making it highly reactive. The “Molecule of the Year” in 1992 [3, 4], the discoveries made about NO and heart function earned Robert F. Furchgott, Louis J. Ignarro and Ferid Murad the Nobel Prize in 1998 (NobelPrize.org). In 1980, endothelium-dependent vessel relaxation was first described [5] and in 1987, it was determined that the endothelium derived relaxing factor (EDRF) was in fact NO [6, 7].

NO moves easily in and out of cells so it cannot be stored inside producing cells like other endogenous messengers. In the presence of oxygen, it has a half life of just a few seconds, but its longevity in the body is not known [1, 2]. There are several nitrogen-derived compounds that exert distinct biological functions including NO+ (nitrosonium), NO· (nitric oxide) and NO− (nitroxy anion). Most studies of nitric oxide do not differentiate or define the specific species that is exerting biological actions [2]. From this point forward, our discussions will be related to NO in general terms.
NO functions via multiple mechanisms to exert biological effects. It reacts directly or indirectly with proteins, lipids, nucleic acids, metals, other gases (such as oxygen) and carbohydrates [1]. Like many other molecules, the effects of NO can be either positive or negative depending on the cellular environment and amounts produced. Both a lack of NO and an excess of NO can lead to pathological consequences such as hypertension and septic shock, respectively [3]. NO is a signaling agent and has been implicated in a broad number of functions including neurotransmission, memory, host defense, vasodilation, blood flow, respiration, nutrient metabolism and apoptosis [3, 8-10]. Therefore, the chemical simplicity of this molecule has no bearing on its broad and essential functions.

The Citrulline-Nitric Oxide Cycle

The citrulline-NO cycle, comprised of the enzymes argininosuccinate synthase (AS), argininosuccinate lyase (AL) and endothelial nitric oxide synthase (eNOS), is responsible for the regulated production of nitric oxide [11]. AS is a homotetrameric enzyme with a molecular weight of ~47 kDa per monomer. Each monomer contains 412 amino acids. It is transcribed from a single gene on chromosome 9 and is expressed in virtually all tissues with the highest expression in liver, kidney and brain [12-17]. AL is also a homotetramer with a subunit molecular weight of ~50 kDa. The human AL gene is found on chromosome 7 and has 464 amino acids per monomer. It is expressed widely, similar to AS [13, 17, 18]. There are 3 isoforms of nitric oxide synthase (NOS): neuronal NOS (nNOS; NOS1), inducible NOS (iNOS; NOS2) and endothelial NOS (eNOS;
NOS3). Each is encoded by a separate gene on chromosomes 12, 17 and 7, respectively, and they share ~ 50-60% sequence homology [19, 20]. eNOS, the NOS isoform studied in this work, is a homodimer with a molecular weight of 135 kDa per subunit that is expressed in endothelium, skeletal and cardiac muscles, kidney tubules and many other non-endothelial tissues [21, 22]. eNOS, like nNOS, is a constitutive NOS isoform that is regulated by calcium/calmodulin. This is in contrast to iNOS which is active even at low calcium concentrations [19]. Besides the substrates necessary to carry out its reaction (see Table 1), eNOS requires tetrahydrobiopterin (BH4), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and heme as co-factors. The amounts of NO produced by constitutive isoforms is ~ 1000 fold less than iNOS [19, 20].

The following are the reactions catalyzed by each of the enzymes in the endothelial citrulline-NO cycle:

<table>
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<th><strong>Table 1: Reactions Catalyzed by Citrulline-NO-Cycle Enzymes.</strong></th>
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<td><strong>eNOS (EC 1.14.13.39):</strong></td>
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<td>L-arginine + NADPH + H + O2 = L-citrulline + NO + NADP+</td>
</tr>
<tr>
<td><strong>AS (EC 6.3.4.5):</strong> (rate limiting step)</td>
</tr>
<tr>
<td>ATP + L-citrulline + L-aspartate = AMP + diphosphate + argininosuccinate</td>
</tr>
<tr>
<td><strong>AL (EC 4.3.2.1):</strong></td>
</tr>
<tr>
<td>argininosuccinate = fumarate + L-arginine</td>
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</tbody>
</table>
The function of the citrulline-NO cycle as a whole is crucial for the production of NO. The availability of arginine is a key factor limiting NO synthesis, despite the fact that intracellular concentrations of arginine (0.1-0.8 mM) are greatly in excess of the reported eNOS Km (~5 µM) [11]. This phenomenon is termed “the arginine paradox”. Although there are transport systems available that have been hypothesized to supply the arginine utilized for NO production [23], we and others have shown that endothelial NO production is limited by the capacity to regenerate arginine from citrulline [24-27]. In fact, cells that express constitutive NOS isoforms, such as endothelial cells and neurons have the capacity to regenerate arginine from citrulline [25, 28].

Dysfunction of AS and AL, which are also part of the urea cycle in ureagenic tissues, can lead to metabolic defects termed collectively as urea cycle disorders, since deficiency in any one of the six enzymes in the urea cycle causes disease [29]. Type I citrullinemia is an autosomal recessive disorder caused by AS deficiency (OMIM #215700) [30]. Some of the symptoms of this disease include severe vomiting, excess levels of citrulline in serum, spinal fluid and urine, hyperammonemia and mental retardation. If not treated promptly, it can lead to death. Death in the neonatal period occurs in nearly half of cases [29]. Many mutations have been identified in the AS gene that can lead to type I citrullinemia [31, 32].

Unlike classical citrullinemia in children that results from a mutation in the AS gene and is associated with an overall deficiency of AS, in type II citrullinemia there is no mutation in the AS gene (OMIM #603471) [33]. AS protein has normal kinetic
properties and is quantitatively deficient only in the liver. The gene that is actually
defective is SLC25A13. It encodes a mitochondrial Ca\(^{2+}\)-dependent aspartate/glutamate
transporter called citrin [33, 34]. The loss of organization attributable to the mutated
citrin leads to reduction of AS protein, possibly through destabilization and/or
degradation. Symptoms of type II citrullinemia include enuresis, delayed menarche,
insomnia, nocturnal sweats, recurrent vomiting, diarrhea, tremors, episodes of confusion
after meals, lethargy, convulsions, delusions, hallucinations, and brief episodes of coma.
Some patients die within a few years of onset [29]. For both type I and type II
citrullinemia, a low protein diet, medications to reduce ammonia levels (examples:
sodium benzoate, sodium phenylacetate) or dialysis can be used as treatments to reduce
ammonia levels [35].

Argininosuccinic aciduria is an autosomal recessive disorder caused by multiple
possible mutations in the AL gene (OMIM 207900) [36]. Like citrullinemia,
argininosuccinic aciduria can be early onset (more severe) or late onset. Some symptoms
of early onset AL deficiency include lethargy, skin lesions, mental retardation, liver
enlargement and convulsions. Late onset disease is usually mild and symptoms tend to
occur during illness or periods of stress. Treatment for argininosuccinic aciduria is the
same as for citrullinemia [35].
Endothelial Nitric Oxide and Vascular Health

NO regulates most normal functions of the endothelium. One primary function of NO is vasodilation. This process occurs when NO produced in the endothelium migrates to the adjacent smooth muscle layer, binds to the heme moiety of soluble guanylyl cyclase (sGC) and activates it. This leads to an increase in cyclic guanosine monophosphate (cGMP), which then activates protein kinase G (PKG) and leads to vasodilation. Regulation of vessel dilation contributes to blood flow and blood pressure control [37, 38].

In addition, NO regulates platelet aggregation. The health of arteries is highly dependent on tight control over this process. During injury, platelets migrate to the site of injury and form a plug to seal the blood vessel and minimize blood loss. On the other hand, uncontrolled platelet aggregation diminishes the fluidity of blood, prevents the delivery of oxygen and nutrients to tissues and can lead to thrombosis- a leading event in myocardial infarction. During physiological conditions, the balance is tipped towards reducing platelet aggregation. Both endothelial and platelet derived NO lead to the activation of PKG, the inhibition of cAMP phosphodiesterase and the reduction of cytosolic calcium. This leads to decreased platelet aggregation and adhesion and a disaggregation of existing platelet aggregates [39, 40].

Another key vascular function regulated by NO is angiogenesis, which is defined as the formation of new blood vessels. If unchecked, this process can be pathogenic.
Physiological angiogenesis is important for improving ischemic conditions and for wound healing [41, 42]. Vasodilation is required for angiogenesis as evidenced by the fact that many angiogenic factors, such as VEGF, possess vasodilating properties [43, 44]. NO has been demonstrated to have a direct role in angiogenesis since NO donors promote and NO inhibitors diminish angiogenesis [45-48]. NO promotes angiogenesis via cGMP elevation and by enhancing the expression of angiogenic factors [41, 42, 48, 49]. Enhancement of NO production in patients undergoing coronary artery bypass grafting by administration of L-arginine in conjunction with VEGF improved angiogenesis and surgical outcomes, thus exemplifying the tight association between NO signaling and angiogenesis [50].

NO can be both pro-and anti-apoptotic. High concentrations of NO induce cell death in macrophages, pancreatic islet cells, tumors and other cell types. There are several mechanisms by which NO can promote apoptosis. First, NO can activate the mitochondrial apoptosis pathway by increasing the release of cytochrome c. This leads to activation of the caspase-dependent apoptosis pathway. NO can also cause DNA damage which induces p53 and leads to cell cycle arrest. Excess NO activates pro-apoptotic kinase cascades such as c-Jun N-terminal kinase and mitogen activated protein kinases. Finally, NO can cause an increase in the release of ceramide, a bioactive sphingolipid that can itself induce apoptotic pathways via the death receptor pathway [51-54]. Cytokines, high glucose lipids mediate some of the pro-angiogenic properties of NO [55-60]. NO deficiency also leads to apoptosis [51-54].
On the other hand, physiological concentrations of NO protect cells from apoptosis [51-54]. One mechanism of protection is via the release of cGMP, which subsequently diminishes cytochrome c release, caspase activation and ceramide accumulation. In addition, NO can lead to suppression of caspase activity by reversible S-nitrosylation and increase the expression of anti-apoptotic genes such as Bcl-2 and HSP70 [54]. Shear stress is an important mechanism that protects endothelial cells from TNFα or growth factor withdrawal-induced apoptosis [61]. One mechanism proposed for this protective effect is via the upregulation of NOS and superoxide dismutase [62].

Considering the plethora of vascular functions regulated by NO, any dysregulation of its bioavailability and production has far reaching consequences. In fact, endothelial dysfunction is associated with a number of disorders including diabetes, obesity, atherosclerosis, metabolic syndrome and hypertension [63-67]. Much of what is known related to endothelial dysfunction and impaired NO production is related to the expression, activation, localization, interactions and substrate/cofactor availability of eNOS [63]. The role of other citrulline-NO cycle components remains relatively unexplored.

Endothelial Dysfunction

There are a number of mechanisms that have been implicated as contributing events in endothelial dysfunction, and most are related to impaired function of NO. One hallmark of endothelial dysfunction is inflammation [68]. NO possesses anti-
inflammatory properties that are disrupted in endothelial dysfunction via mechanisms involving increased leukocyte adhesion, increased expression of chemokines, increased monocyte migration and infiltration of monocytes into the arterial wall [65, 69]. Another inflammatory process disrupted in endothelial dysfunction is related to an increased level of C-reactive protein (CRP). CRP diminishes the production of NO by reducing eNOS expression [69]. Multiple other inflammatory molecules are elevated in endothelial dysfunction such as TNFα. Whether elevated TNFα is a cause or consequence of endothelial dysfunction is debatable. However, there are multiple mechanisms by which TNFα causes endothelial dysfunction such as the inhibition of insulin signaling, an increase in lipids such as ceramide and increased apoptosis [70-77].

In endothelial dysfunction, there is also an increase in reactive oxygen species (ROS). This is one central mechanism by which cardiovascular disease risk factors such as diabetes, hypercholesterolemia and hypertension lead to endothelial dysfunction [63]. One such species, superoxide, reacts with NO and leads to the production of peroxynitrite, a toxic free radical that diminishes the bioavailability of NO and inhibits the protective actions of NO [78]. ROS also degrade BH4, an important eNOS co-factor. This leads to the uncoupling of NADPH oxidation from eNOS-mediated NO synthesis and the subsequent production of superoxide instead of NO [79-81].

Another consequence of increased ROS is the inhibition of dimethylarginine dimethylaminohydrolase (DDAH). This leads to increased levels of asymmetric dimethylarginine (ADMA), an endogenous eNOS inhibitor [82]. ADMA inhibits eNOS
by multiple mechanisms such as further increasing superoxide production and eNOS uncoupling, diminishing the interaction of HSP90 with eNOS and redistributing eNOS to the mitochondria with subsequent mitochondrial dysfunction [83-86]. ADMA has been linked to vascular disease by contributing to reperfusion injury and increasing blood pressure [84, 87]. Protective effects at early time points have also been shown via iNOS mediated inhibition of intima-media thickness [88].

Elevated lipids also influence the function of the endothelium. For example, oxidized LDL reduces eNOS activity by displacing it from the plasma membrane and targeting to intracellular sites where it is less active [89]. Elevated ceramide levels due to excess saturated fat in the diet or chronic inflammation diminish eNOS activation and NO production [60, 90, 91]. Another lipid linked to endothelial dysfunction is apolipoprotein CIII (apoCIII), a component of very low density and low density lipoprotein that is elevated in insulin resistance and metabolic syndrome. ApoCIII activates pro-inflammatory atherogenic pathways by directly inhibiting insulin signaling and reducing the activation of eNOS [92]. In fact, one of the benefits of statin therapy beyond cholesterol lowering is the improved function of eNOS and endothelial function. One example is improved blood flow to ischemic tissues in diabetes in response to statins due to an upregulation of eNOS [93]. There are controversies about whether statins improve endothelial function and this is perhaps associated with other factors associated with endothelial dysfunction. For instance, simvastatin is able to improve endothelium-dependent vasodilation in patients with low levels of ADMA but is ineffective in patients
with high ADMA. On the other hand, arginine supplementation in combination with statins works well with patients with high but not low ADMA [94].

Insulin resistance and diabetes are also tightly associated with endothelial dysfunction [73]. In fact, macrovascular complications are quite common in diabetic patients leading to the classification of diabetes as a vascular disorder [95]. Endothelial cells express insulin receptors and many cardioprotective signaling pathways are mediated by insulin [95]. Several derangements can occur when insulin function is compromised including an increase in ROS, depletion of BH4, lipid abnormalities and hypertension [96-100]. Hyperglycemia, a consequence of diminished insulin action, also has direct impacts on eNOS expression and activity and enhances endothelial cell apoptosis [58, 101, 102]. Collectively, these imbalances impair the ability of the endothelium to produce adequate amounts of NO and all normal endothelial functions are compromised leading to a striking association between insulin resistance, diabetes and coronary artery disease.

Overall, the mechanisms that lead to endothelial dysfunction are numerous and complex. Most of them converge and lead to one major issue- a decrease in the production and bioavailability of NO. Therefore, a better understanding of the mechanisms that control NO production is essential for the development of effective prevention and treatment strategies for vascular disorders.
Prevention of Vascular Disorders

There are multiple medical and surgical approaches to treat cardiovascular diseases and they should be employed as early as possible to diminish long-term damage. Considering the far-reaching consequences of endothelial dysfunction and the fact that by the time significant symptoms arise, vascular disease has already progressed dramatically, prevention strategies are the best insurance against the development of vascular disease [67]. Since most risk factors for heart disease mediate their deleterious effects by impairing NO production [63], preventable risk factors need to be addressed either with lifestyle modification of medical interventions. Healthy eating and maintaining a normal body weight can go an extremely long way towards keeping blood pressure within normal limits, preventing diabetes and metabolic syndrome and maintaining a healthy level of blood lipids [103, 104]. Cigarette smoking directly impairs NO bioavailability [105], so avoiding smoking or quitting is essential. In addition, blood pressure and cholesterol, if inadequately responsive to lifestyle measures, should be treated pharmacologically [106].

Argininosuccinate Synthase Functions Related to Nitric Oxide Production

AS, the focus of this dissertation, has several unique properties that are essential for NO production and vascular health. AS is rate limiting for the production of NO [107]. This was initially demonstrated in vascular smooth muscle cells when AS overexpression enhanced the capacity of transfected cells to produce NO, despite non-
limiting levels of arginine [108]. In endothelial cells, we have demonstrated that inhibition of AS activity with siRNA or the AS inhibitor α-methyl-DL-aspartic acid diminishes NO production due to reduced AS activity [24, 109].

We also demonstrated that the co-fractionation of AS, AL and eNOS in plasmalemmal caveolae allowed endothelial cells to effectively distinguish “bulk intracellular arginine” from the arginine used for NO production [110]. Our substrate utilization studies supported this view by demonstrating that the recycling of citrulline back to arginine is an efficient, tightly coupled process [24]. The activity of AS in this recycling process is the preferred mechanism for eNOS-mediated NO production, while iNOS-mediated NO production in smooth muscle is dependent on arginine transport [26].

One additional and unique mechanism of regulation of NO production by AS involves expression of endothelium-specific AS variants with different lengths of the 5′ untranslated region (5′ UTR). The shortest form of the message represents ~90% of the total AS mRNA and encodes for full length AS. The two longer forms contain an out of frame upstream open reading frame (uORF) that encodes a 4 kDa protein called Argininosuccinate Synthase Regulatory Protein (ARP). Overexpression of ARP leads to diminished NO production and siRNA knockdown of ARP leads to an increase in NO production in endothelial cells [111].

The three dimensional structure of human AS has revealed an interesting feature relevant to its role in NO production. It has been demonstrated that nitrosylation of AS at
cysteine 132 (C132) leads to inactivation of the enzyme [112]. A mechanism was not identified for this activation. The investigators who solved the human AS crystal structure used in silico modeling to predict the effect of nitrosylation at this site on the catalytic efficiency of AS. The authors noted that C132 is actually buried in the N-terminal domain. They suggested that a significant structural change would have to take place for the nitrosylation to occur. Based on their in silico models, they determined that even though C132 is not near the active site, the modification does perturb the orientation of amino acids that are part of the active site [113]. This presents the first evidence of a possible conformational change caused by a modification at an allosteric site which then changes the activity of AS with subsequent effects on NO synthesis.

Regulation of Endothelial Nitric Oxide Production: Protein Expression

The functions of NO are controlled at the levels of biosynthesis and availability because it cannot be stored. Since virtually all phenotypic properties of normally functioning endothelial cells are related to NO, its production is tightly controlled [28, 114, 115]. This tight control requires multiple regulatory mechanisms. One such mechanism is the regulation of the expression of the enzymes of the citrulline-NO cycle.

To assess the importance of the expression citrulline-NO cycle components on vascular function, animal knockout models have been generated and have yielded important information. The eNOS knockout mouse models have by far been the most prominent in delineating the importance of the citrulline-NO cycle for vascular health.
Deletion of the eNOS gene is not embryonic lethal, but does lead to hypertension, impaired wound healing and angiogenesis [116]. In addition, eNOS knockout mice have hyperinsulinemia, hyperlipidemia, diminished glucose metabolism [117] and a decrease in skeletal muscle oxidative capacity [118]. In contrast, eNOS overexpression ameliorates vascular dysfunction by improvements in lipids, blood pressure, myocardial contraction and protection against ischemia-reperfusion injury [119, 120]

The lessons we have learned from eNOS knockout mice are complicated by the fact that in some instances, a lack of eNOS has some beneficial effects and overexpression of eNOS has deleterious effects. For example, eNOS knockout mice have decreased formation of diet-induced fatty streaks [121] and endothelium dependent relaxation and bradykinin-mediated blood flow are intact due to a compensatory upregulation of nNOS [122]. Although physiological levels of NO are beneficial for myocardial contractility, overexpression leads to a negative inotropic effect [123]. Due to the difficulty in interpreting mouse data related to compensatory functions of the NOS isoforms, a triple knockout mouse where eNOS, nNOS and iNOS were deleted was recently generated. These mice had a much lower survival rate and displayed a long list of metabolic abnormalities including metabolic syndrome, obesity, hyperlipidemia, hypertension, and impaired glucose tolerance [124]. Taken together, animal models of reduced or enhanced eNOS expression demonstrate the essential role of eNOS in mediating vascular health.
In contrast, when AS is knocked out in mice, it leads to death a few days after birth due to the accumulation of citrulline and a severe arginine deficiency [29, 125]. A tissue specific AS knockout mouse model is not available, but if, for example, such a model were generated with an endothelium specific deletion of AS, we would predict marked endothelial dysfunction based on our tissue culture work. Similarly to the AS knockout, AL knockout mice die within 48 hours of birth and are characterized by increased ammonia, argininosuccinic acid, glutamine and citrulline and low levels of arginine [29, 36].

The role of the expression citrulline-NO cycle components on vascular health can be studied at extreme levels utilizing animal models as just described. These scenarios are rare in humans, and there are finer levels of regulation that occur in response to a number of biological processes and signals. Shear stress is one of the most important physiological factors that regulates the function of blood vessels [126-128]. Unidirectional, laminar shear stress exerts a protective role on the endothelium by diminishing adhesion and platelet aggregation [129] and enhancing NO production [126, 130]. Shear stress has been shown to increase eNOS expression via a transient activation of NfkB in bovine aortic endothelial cells. There is a shear stress responsive element (SSRE) in the eNOS promoter that is responsible for the transcriptional regulation by NfkB [131]. Other groups have determined that the shear stress-mediated increase in eNOS mRNA is due to an increase in transcription and mRNA stabilization [132, 133]. In a DNA microarray study of human umbilical vein endothelial cells, shear stress was shown to significantly increase AS expression, but not eNOS. The authors suggested this
might be due to the fact that shear stress-induced increases in NO synthesis depend on arginine synthesis from citrulline via increased AS levels [134]. In a subsequent study by a group studying the adhesion of endothelial cells in vein grafts supported the role of both eNOS and AS in the response of the endothelium to shear stress [135]. The role of AL in shear stress-mediated NO production has not been studied. However, it is clear that there is a role for both AS and eNOS in mediating the response of the endothelium to shear stress that involves multiple and perhaps overlapping mechanisms depending on the level of shear stress and other factors.

Cytokines regulate vascular function in various ways, and tumor necrosis factor alpha (TNFα) is well characterized in this respect. TNFα is a pro-inflammatory cytokine with both positive and negative roles in regulating vascular function, depending on the length of treatment and tissue type [136]. In general, conditions of chronic TNFα elevations are deleterious to the vasculature as evidenced by the fact that humans with diabetes, obesity and heart disease have elevated levels of TNFα [55, 71, 73, 75, 137, 138]. Often times, TNFα-mediated vascular impairment is caused by dysregulation of NO production [70, 74, 139, 140]. One mechanism by which TNFα inhibits NO production is by altering eNOS and AS expression. Specifically, eNOS expression has been shown to be downregulated by TNFα via several mechanisms including decreased mRNA stability and inhibition of transcription factor binding [141-143]. In addition, TNFα diminishes AS expression and NO production by inhibiting the binding of SP-1 elements to the proximal promoter [144]. AL seems to be less amenable to cytokine regulation since in vascular smooth muscle cells, retinal endothelium or pancreatic β cells, treatment with a variety
of different cytokines led to co-induction of AS and iNOS while AL remained static [145-147].

Peroxisome proliferator-activated receptor gamma (PPARγ) is a member of the nuclear receptor superfamily of ligand-activated transcription factors. PPARγ regulates the transcription of genes involved in lipid and glucose metabolism [148], differentiation [148-150] and cell growth [148]. Thiazolidinediones (TZDs) are a group of synthetic PPARγ agonists that provide cardiovascular benefits such as reduction of blood pressure and improvement in insulin-sensitivity [151-153]. In addition, TZDs reduce lesion formation in animal models of atherosclerosis [154-156], improve flow-mediated vasodilation and decrease vascular smooth muscle cell migration by stimulating endothelial NO production [157, 158]. These anti-diabetic compounds also counter the effects of the inflammatory response associated with elevated serum levels of TNFα and ceramide. For instance, rosiglitazone impairs TNFα-induced activation of MAPK, restoring insulin signaling and leading to normalization of glucose uptake in brown adipocytes [159]. In humans with type 2 diabetes, pioglitazone was protective against TNF-mediated endothelial dysfunction [76]. In hepatocytes made insulin resistant via TNFα treatment, troglitazone is able to restore insulin sensitivity, partially via blocking the downstream actions of ceramide [160].

Another mechanism by which TZD’s improve endothelial function is by inducing the expression of components of the citrulline-NO cycle. For example, eNOS expression and NO production are enhanced by troglitazone by a mechanism that seems to be PPAR
independent [161]. Telmisartan, an angiotensin II receptor blocker, has recently been identified as a partial PPARγ agonist. Its cardioprotective mechanism is mediated partially by the upregulation of eNOS [153]. In addition, troglitazone enhances NO production and AS expression by activating a PPAR responsive element in the distal AS promoter [162]. There have been no studies on the effects of PPAR agonists on AL. Taken together, the positive benefits of TZD’s are multifactorial and lead to improved vascular parameters by mechanisms that include the upregulation of eNOS and AS.

Insulin is an important regulator of vascular function. Insulin mediates its effects on vasodilation by stimulating NO release [163, 164]. One mechanism by which insulin increases NO production is by increasing the expression of eNOS. Kuboki et al. demonstrated that insulin increases eNOS mRNA within 1 hour of treatment. This increase was mediated by activation of the PI3-kinase pathway [165]. In another study, the mechanism of insulin transcriptional regulation of eNOS was found to involve increased binding of SP-1 and AP-1 transcription factors [166]. The link between insulin and NO has another important feature. NO itself leads to the secretion of insulin in pancreatic islet β-cells [167]. This process is dependent on the recycling of arginine to citrulline [168]. This suggests an involvement of both AS and AL. In addition, eNOS expression and function is diminished in diabetes. For example, high glucose diminishes the expression of eNOS [102]. In addition, insulin enhances the expression of eNOS in diabetic rats [169]. The impact of insulin or diabetes on endothelial AS and AL expression has not been studied, but one group did note that in the early phases of streptozotocin (STZ)-induced type 1 diabetes in rats, AS and eNOS mRNA and protein
expression was induced in aorta. This increase then diminished as diabetes progressed. There was very little effect on AL mRNA, and possibly a slight induction after 4 weeks, but the effects on AL were not studied any further [170]. Thus, glucose, insulin and NO metabolism are tightly associated processes that involve both AS and eNOS (and possibly AL) expression.

VEGF is an important mediator of vascular health by regulating vasodilation and angiogenesis [171]. VEGF exerts its cardioprotective functions, in part, by increasing the expression of eNOS. In human umbilical vein endothelial cells (HUVECs), VEGF augments the expression of eNOS in a time and dose-dependent manner. This was linked to increased basal and stimulated NO production [172]. Another group obtained similar results when using rat aortic rings [173]. In addition, insulin increases the expression of both eNOS and VEGF in diabetic rats leading to increased NO production and vascular relaxation [169]. The role of VEGF in regulating AS or AL has not been studied. Therefore, VEGF and NO signaling pathways are linked by direct effects on eNOS expression.

Ceramide, a bioactive sphingolipid, has been implicated in the development of insulin resistance and atherosclerosis [57, 61, 77, 174]. In general, supraphysioloical levels of ceramide contribute to endothelial dysfunction [60, 91, 175] and most of what is known involves impairment of eNOS activation (which is discussed in the next section). However, Li and colleagues demonstrated that ceramide decreases NO production in human endothelial cells via a mechanism that involves ROS generation. They found that
eNOS expression is enhanced as a compensatory mechanism. This enhanced expression was not sufficient to ameliorate the diminished NO synthesis caused by the generation of ROS [90]. The direct effects of ceramide on AS and AL expression have not been studied.

The intricate and diverse mechanisms regulating the expression of AS and eNOS in the endothelium and other tissues suggests that this is an important mechanism mediating vascular biology and warrants continued investigation. For this reason, the regulation of AS expression in endothelial cells encompasses Specific Aim 1 of this dissertation and is described in Chapter One.

*Regulation of Endothelial Nitric Oxide Production: Post-Translational Modifications*

Although controlling the expression levels of AS, AL and eNOS is an important mechanism for regulating the levels of NO produced, this mode of regulation seldom occurs quickly enough to acutely increase or decrease NO production. Since NO is constantly being produced and its levels are frequently adjusted to meet cellular demands, post-translational modifications (PTM) have been described as a prominent mechanism in regulating NO production.

The acute regulation of eNOS by reversible phosphorylation is well described. Although eNOS is regulated by tyrosine phosphorylation [176, 177], serine/threonine phosphorylation is a much more prominent mechanism. There are 5 well characterized
sites of eNOS phosphorylation: serine 116 (S116), threonine 497 (T497), S617, S635 and S1179 (bovine; these sites are equivalent to S114, T495, S633 and S1177 in human) [178-180].

S116 is located in the oxygenase domain of the enzyme [179]. There is controversy as to whether this site activates or inactivates eNOS. A recent paper demonstrated that mutation of this site to an alanine increased NO production while mutation to an aspartic acid diminished NO production. In addition, phosphorylation at this site increased the inhibitory interaction of eNOS with caveolin-1 and diminished vascular reactivity. Hence, this site seems to be inhibitory for the function of eNOS [181]. On the other hand, Drew et al. found that HDL and Apo A1 led to increased NO release due to eNOS phosphorylation at several sites, most profoundly at S116. This suggested a positive role for S116 [182]. It is possible that there are tissue and stimulus-specific functions for S116.

There is a great deal of consensus regarding the inhibitory role of T497, which is located in the eNOS calmodulin binding domain [179]. In fact, in the study mentioned above where they found that S116 does activate eNOS, they also found that HDL and Apo A1 diminish phosphorylation at T497 [182]. Many other studies have confirmed this inhibitory regulation [81, 183-185]. In particular, several different groups have demonstrated that mutation of T497 to aspartic acid (phospho-mimetic) greatly diminishes eNOS activity and NO production while the alanine mutation (phospho-null) has opposite effects [79, 184]. When this site is phosphorylated, calmodulin cannot bind.
to and activate eNOS [184]. T497 is constitutively phosphorylated and upon stimulation with agents that increase intracellular calcium, such as bradykinin or VEGF, the site is dephosphorylated by protein phosphatase 1 (PP1) leading to calmodulin binding [184]. In addition, the dephosphorylation of T497 has to occur before the activating phosphorylation at S1179 can take place [185]. Protein kinase C has been shown to increase phosphorylation at this site [183], thereby diminishing eNOS function. Certain PKC isoforms, such as PKCα and PKCε can activate eNOS by leading to phosphorylation at S1179 [186, 187].

Serines 617 and 635 are both in the eNOS autoinhibitory domain, which is part of the reductase domain [179]. This domain is reported to keep eNOS in an inhibited state that is reversed upon calmodulin binding [19, 188]. These sites are phosphorylated in response to bradykinin, ATP and VEGF [179, 182, 189]. Shear stress has also been found to lead to the phosphorylation of S635 [190]. The phosphorylation at S617 is transient in nature while S635 phosphorylation is more persistent. Akt phosphorylates S617 while PKA phosphorylates S635. The mechanism of regulation of eNOS activation by these two sites is suggested to involve a two step process. During initial agonist-induced activation of eNOS, S617 is phosphorylated and increases eNOS sensitivity to calcium. Then, S635 phosphorylation increases eNOS specific activity to levels similar to what occurs when S1179 is phosphorylated [189].

The most studied site of eNOS phosphorylation is S1179, located near the C-terminus in the reductase domain [179]. Phosphorylation at this site leads to an
approximately 2-fold activation of eNOS specific activity [189]. This activation is due to a decrease in the calcium-dependence of eNOS and an increase in electron flux from the reductase domain to the oxygenase domain, which leads to increased NO output [191]. This site is phosphorylated by a number of kinases including Akt, AMPK, PKA, PKG and calmodulin II protein kinase in response to a variety of stimuli including insulin, adiponectin, bradykinin, VEGF, HDL and Apo A1 [127, 130, 182, 183, 185, 186, 192-202]. For example, fluid shear stress, VEGF and bradykinin lead to the activation of PKA and subsequent phosphorylation of eNOS at S1179 [183, 203].

Although the regulation of eNOS by a complex pattern of stimulus-specific phosphorylation and dephosphorylation events has been studied extensively, there have been no reports of such studies for AS and AL. Evidence supporting post-translational regulation of AS is beginning to accumulate. First, in a proteomic study to identify novel phosphoproteins in HeLa cells, AS was found to be phosphorylated at S352 [204]. This is the first and only indication of this type of modification of AS. The authors did not investigate the biological relevance of this modification. In addition, in vascular smooth muscle, AS is reversibly inactivated by nitrosylation in conditions of high NO output by iNOS. Their work suggests that AS is at least partially responsible for sensing cellular NO levels and adjusting output accordingly in an effort to maintain homeostasis [112]. Nitrosylation of cysteine residues has emerged as an extremely important, reversible modification that regulates the activity of a number of proteins such as caspase-3, the ryanodine receptor and actin [205]. Importantly, eNOS is basally nitrosylated in endothelial cells and is then rapidly denitrosylated in response to VEGF stimulation
The nitrosylation of AS in the endothelium has not been studied, but may represent an important additional mechanism of regulation.

Another important PTM that regulates NO production is the acylation of eNOS by the irreversible N-myristoylation at glycine 2 (G2) and the reversible thiopalmitoylation at cysteine 15 (C15) and C26 [207]. These modifications are crucial for the targeting and anchoring of eNOS to the plasma membrane, which is an essential location for the production of NO [207-210]. These modifications have not been studied for AS or AL. However, we have demonstrated that AS and AL and eNOS co-fractionate with caveolin-1 in caveolar membrane fractions, which indicates that mechanisms must exist to target these enzymes to the plasma membrane in conjunction with eNOS.

Glycosylation is an additional type of PTM that regulates NO production. O-linked glycosylation occurs at serine and threonine residues, typically in response to the flux of excess glucose into the hexosamine biosynthesis pathway. Glycosylation often competes with phosphorylation at the same or adjacent sites leading to a ying-yang type of regulatory scheme [211, 212]. In endothelial cells, glycosylation of eNOS decreases its activity and diminishes phosphorylation at S1179 [213]. The functional relevance of this modification was illustrated in diabetes-related erectile dysfunction, where glycosylation reduced S1179 phosphorylation at baseline and in response to VEGF and shear stress [214]. In this study, it was determined that glycosylation only affects S1179 and not T497, S617 or S635. Although glycosylation of AS in endothelial cells has not been studied, in Caco-2 cells the expression of AS is stimulated by glutamine via glycosylation
of the transcription factor SP-1 [215]. This modification leads to nuclear import of SP-1 and a subsequent increase in AS transcription. The regulation of AL by glycosylation has not been studied.

Taken together, the extensive studies of eNOS PTM by phosphorylation, nitrosylation, glycosylation and acylation have a prominent role in regulating the ability of the endothelium to produce NO. Due to the few papers in the literature that suggest that AS is also regulated by PTM and to the prominent role of serine/threonine phosphorylation in regulating eNOS function, Specific Aim 2 is dedicated to the thorough investigation of endothelial AS serine/threonine phosphorylation. The findings are presented in Chapter Two.

*Regulation of Endothelial Nitric Oxide Production: Subcellular Localization and Protein Interactions*

The precise location of a protein within the cell is critical for its function. Compartmentalization of proteins that are in the same metabolic or signaling pathway allows for efficient communication, channeling of substrates and accessibility to important regulatory factors. In addition, the regulation of subcellular transport to localize these proteins involves several mechanisms including vesicles, cytoskeletal components and protein interactions. Furthermore, protein interactions often mediate the functions of their interacting partners via mechanisms that do not involve transport such as post-translational modification or conformational change [216]. There is a complex
network of interacting proteins that are known to regulate NO production via eNOS [180] and the scientific community is just beginning to describe similar mechanisms for AS and AL.

The regulation of eNOS is dependent its localization within the cell [217, 218]. There are two regions in the cell that have been found to independently produce NO, the Golgi and the plasma membrane [219, 220]. Within the plasma membrane, eNOS is localized to caveolae [217]. Caveolae are invaginations of the plasma membrane that are rich in glycosphingolipids and cholesterol and serve as platforms for the integration of signaling pathways. Caveolae are also involved in endocytosis and transcytosis. Caveolin is an integral membrane protein that is an important structural component of caveolae [218, 221]. Although activation of eNOS by phosphorylation at S1179 does not cause translocation from the Golgi to the plasma membrane, the proper localization of eNOS is necessary for this phosphorylation to occur [208, 220]. Mistargeting of eNOS also attenuates NO release [222, 223]. In the plasma membrane, eNOS is constitutively phosphorylated at S1179 and is highly active. In the Golgi, phosphorylation at S1179 is diminished and less NO is produced in response to calcium signaling [224]. Furthermore, plasma membrane eNOS is more responsive to agonists such as insulin and angiopoietin and demonstrates increased binding to HSP90 as compared to Golgi-eNOS [219]. Despite this understanding, there is still some doubt as to whether Golgi versus plasma membrane NO production is of more or less significance or whether the distinct functions of these pools require further study to define their specific purpose in the cell.
The targeting of eNOS to Golgi versus plasma membrane is controlled by several mechanisms. One key mechanism involves myristoylation and palmitoylation [209]. Myristoylation is necessary for eNOS to associate with the membrane, essential for optimal eNOS activity, important for compartmentalization in the Golgi and is a prerequisite for palmitoylation. Palmitoylation is essential for eNOS targeting to the membrane and specifically to caveolae [210]. In a study by Jagnandan and colleagues, organelle-specific eNOS constructs were generated in an attempt to determine the compartment-specific function of eNOS. The authors found that the nucleus, mitochondria cytoplasm and trans-Golgi network are inefficient for NO production as compared to the plasma membrane and cis-Golgi network. This is apparently not due to insufficient substrate availability since iNOS targeted to the same regions functions normally. The relative lack of calcium/calmodulin in these regions may responsible for the inefficiency of eNOS, which unlike iNOS is dependent on calcium and calmodulin for function [224]. A subsequent study determined that calcium and calmodulin are not the reason for disparate function in different organelles. The study found it is actually phosphorylation that regulates organelle-specific eNOS function since Golgi localized eNOS had reduced phosphorylation at S1179, S635 and S617 [225].

In addition to acylation as a targeting mechanism, there is an intricate network of cytoskeletal components that regulate eNOS function and likely also its translocation. Actin is a prominent regulator of eNOS. For example, F-actin is required for shear stress mediated eNOS upregulation due to its role in mechanotransduction [226]. In general, the interaction of eNOS with actin increases its activity. One mechanism is due to increased
interaction with HSP90 [227]. On the other hand, the interaction of eNOS with actinin-4, an actin-associated protein, inhibits eNOS activity by preventing calmodulin from interacting with and activating eNOS [228]. Vimentin is another important cytoskeletal component that regulates eNOS function. Vimentin is an intermediate filament that is essential for flow-mediated dilatation [226]. Although the eNOS activity per se has not been related to vimentin, the importance of this cytoskeletal component in mediating vessel relaxation implies the involvement of eNOS. Vimentin is also an essential structural component of caveolae [229]. In addition, the inhibition of microtubules (tubulin and microtubule associated proteins) leads to decreased eNOS activity and NO production [230]. Furthermore, it has been demonstrated that HSP90 and calmodulin both interact with tubulin. The interaction of tubulin with HSP90 is important for eNOS activity since disruption of microtubule assembly diminishes the eNOS-HSP90 interaction and reduces NO output The specific role of the calmodulin-tubulin interaction on the function of the citrulline-NO cycle is unclear [230]. As discussed in a latter section, both HSP90 and calmodulin are important binding partners of eNOS that stimulate its function. This suggests that microtubules also play a role in eNOS function. Whether the mechanism is structural or involves eNOS transport is not clear.

Finally, several protein interactions are essential for eNOS cellular transport. One well studied interaction is with caveolin-1. AS discussed previously, caveolin-1 is an important component of caveolae. It is believed that caveolae function as vesicles that regulate eNOS transcytosis and caveolin-1 is required in this process [222]. More importantly, caveolin-1 and eNOS both interact with NOSTRIN (eNOS trafficking
inducer), which also recruits dynamin, a Golgi localized GTP-ase, and facilitates caveolar transport [231]. NOSTRIN transport of eNOS is dependent on the actin cytoskeleton [232]. Although NOSTRIN is important for eNOS translocation, it inhibits eNOS activity regardless of localization in the cell and overexpression of NOSTRIN leads to redistribution of eNOS from the plasma membrane to intracellular vesicular structures [232]. The specific regulation of this complex and its trafficking is still unclear. NOSIP (eNOS interacting protein) is another important protein that regulates eNOS translocation. NOSIP interacts directly with eNOS and inhibits its activity [233]. Furthermore, NOSIP is important for the subcellular redistribution of eNOS between the caveolae and intracellular compartments [234]. Much like NOSTRIN, NOSIP overexpression leads diminished plasma membrane localization of eNOS [233].

To date, there have been no studies delineating the regulation of AS or AL intracellular targeting. The localization of AS and AL has been understudied in endothelial cells. However, we have demonstrated that AS, AL eNOS and caveolin-1 co-fractionate in caveolar membrane preparations [110]. In vascular smooth muscle, AS demonstrates both cytoplasmic and membrane localization along with a punctuate pattern of distribution that suggests mitochondrial localization [108]. Otherwise, no other studies have addressed the subcellular localization of AS or AL as part of the citrulline-NO cycle. Much more is known about their localization in tissues that produce urea. For example, in liver, these enzymes are localized in mitochondria.
In addition to the role protein interactions play in the targeting of eNOS, there are several proteins that regulate eNOS functions in other ways. Aside from the role of caveolin in AS localization, it has direct effects on inhibiting eNOS activity [235]. The interaction of caveolin with eNOS is direct and occurs in a region of eNOS that contains a caveolin binding motif [235]. The interaction of eNOS with caveolin is disrupted upon stimulation with calcium ionophores, bradykinin or fluid shear stress [236, 237]. Another interaction that negatively regulates eNOS function is the bradykinin receptor. This interaction is similar to that with caveolin in that it represents a membrane docking interaction that is relieved upon treatment with agonists such as bradykinin and calcium ionophores [238].

There are a multitude of proteins that interact with eNOS and enhance its function. One prominent interaction is with heat shock protein-90 (HSP90). This molecular chaperone activates eNOS via a mechanism that involves phosphorylation at S1179 [239]. HSP90 delivers the kinase Akt to eNOS and promotes its phosphorylation [240]. Calmodulin is an important positive regulator of eNOS that also interacts with HSP90 and is responsible for displacing the eNOS-caveolin interaction [241]. In fact, caveolin and calmodulin are involved in the reciprocal regulation of eNOS. Upon agonist stimulation and an increase in intracellular calcium, calmodulin binds to eNOS and displaces caveolin [242]. In addition, eNOS interacts with the CAT-1 arginine transporter and this increases its activation via phosphorylation at S1179 and 635. The mechanism of enhanced NO release does not involve arginine transport [243].
In contrast to eNOS, very little is known about protein interactions involving AS and AL as part of the citrulline-NO cycle. Recently, the interaction of an NADPH sensor protein (HSCARG) with AS was shown to down regulate AS activity in epithelial cells. Their results implied that HSCARG regulation of AS activity is crucial for maintaining the intracellular balance between redox state and NO levels [244]. This is the first study to define a protein interaction with AS that is essential for NO production.

Given the prominence of regulation of NO production by eNOS subcellular localization and protein interactions, we hypothesize similar mechanisms exist to regulate the role of AS in endothelial NO production. Therefore, Specific Aim 3 of this dissertation will explore both the subcellular localization and protein interactions of AS. Our findings are described in Chapter Three.

References


[34] T. Saheki, and K. Kobayashi, Mitochondrial aspartate glutamate carrier (citrin) deficiency as the cause of adult-onset type II citrullinemia (CTLN2) and idiopathic neonatal hepatitis (NICCD), J Hum Genet 47 (2002) 333-341.


SPECIFIC AIMS

Purpose

Heart disease is the number one killer of Americans. As the incidence of obesity, diabetes and metabolic syndrome continues rising at an alarming rate, so will the prevalence of endothelial dysfunction [1]. Since endothelial dysfunction is often accompanied by diminished or excessive nitric oxide (NO) production [2], it is essential to continue our efforts to gain a better understanding of the regulation of NO synthesis. Our studies were designed to expand our global understanding of vascular biology by assessing the regulation of the citrulline-NO cycle from a different perspective. Since much of the focus has been on the regulation of endothelial nitric oxide synthase (eNOS), our research is targeted at a better understanding of another equally important component of the citrulline-NO cycle, argininosuccinate synthase (AS).

Central Question and Hypothesis

AS is an enzyme that is important for the production of nitric oxide. The central question we are addressing with the work described is: How does AS regulate endothelial NO production? Due to the fundamental role of AS for vascular biology, we hypothesize
that multiple mechanisms regulate AS function to control NO production and support endothelial function.

Specific Aim 1

The role of eNOS in the control of NO production is well studied [2]. Much less is known about the regulatory role of AS. We and others have demonstrated the importance of AS expression for the production of NO [3-14]. We hypothesize that a multitude of stimuli alter AS expression to enhance NO production in a manner consistent with the substrate needs of eNOS. In Specific Aim 1, described in Chapter One, we will first examine the role of AS overexpression in regulating NO production. We will then determine whether insulin, vascular endothelial growth factor (VEGF) and ceramide impact AS expression coordinately with eNOS.

Specific Aim 2

Although regulation at the level of transcription and translation is an important mechanism controlling the level of function of an enzyme, this type of regulation seldom accounts for acute changes in enzymatic activity. NO synthesis is a constant and dynamic process. It is well documented that eNOS is regulated by a complex set of reversible post-translational modifications [15-17]. Since AS is the source of substrate for eNOS [9, 11], we hypothesize that a similar pattern of post-translational modifications exists for the acute regulation of AS function. In Specific Aim 2, described in Chapter Two, we will
determine whether AS is an endogenous phosphoprotein, define the biological significance of AS phosphorylation, and uncover the possible mechanisms by which AS phosphorylation regulates its function.

Specific Aim 3

Post-translational regulation of protein function often involves subcellular localization and dynamic protein interactions. There is a complex literature surrounding the regulation of eNOS trafficking, activation and function that is driven by protein interactions [17-19]. However, very little is known about such regulatory mechanisms controlling AS function. In Specific Aim 3, described in Chapter Three, we will define the subcellular localization of AS and identify key interacting partners.

Working Model

It is our belief that the regulation of caveolae-localized AS, in conjunction with eNOS, is mediated by an interrelated set of mechanisms that controls the expression, post translational modifications and protein interactions that are so critical for the overall function of the system. As demonstrated in Figure 1, we hypothesize that agonists and antagonists will coordinately regulate the expression, activation and protein interactions of AS and eNOS so that the level of NO produced is adequate to meet the current needs of the cell. In addition, it is our belief that the multiple mechanisms that regulate AS and eNOS are inter-related, yet specific to environmental signals. Although there might be
instances where there is some discoordinate regulation of AS and eNOS, such instances are necessary in scenarios where an initial change in regulation of one of these two enzymes would signal a need for a change in NO production which would then be followed by a coordinate regulation of the other enzyme for the express purpose of restoring homeostasis. We also believe that there are specific mechanisms that control the regulation of the caveolae-localized citrulline-NO complex in comparison to other locations within the cell. Importantly, the coordinate, multi-level regulation of AS and eNOS is designed specifically to regulate the synthesis of NO in an attempt to support the health of the endothelium and ultimately of the cardiovascular system. Any derangements of the physiological regulation of this complex, even if minor, would have a great impact on vascular health and explains the prominence of vascular disorders seen with a variety of metabolic imbalances.
Figure 1: Working Model of the Regulation of the Citrulline-NO Cycle Under Physiological Conditions. Figure depicts the central components of our study of the citrulline-NO cycle, AS and eNOS, as a caveolae-localized complex that is coordinately regulated by agonists and antagonists via expression, phosphorylation and protein interactions in an effort to produce adequate amounts of NO to promote vascular health.
References


Overview

Argininosuccinate synthase (AS) is a key, regulated step of the citrulline-nitric oxide (NO) cycle. We have previously demonstrated several levels of regulation of AS expression that impact NO production. First, when AS expression is reduced utilizing siRNA, NO production, AS enzymatic activity and endothelial cell viability is diminished. The loss of endothelial cells is via apoptosis and can be rescued with an NO donor, demonstrating the direct role of AS activity and NO production in maintaining endothelial cell viability. In addition, TNFα, a cytokine associated with vascular disease, leads to decreased expression of AS under conditions of chronic inflammation. This decreased expression occurs via a reduction in the ability of SP-1 elements to activate the AS proximal promoter, similar to the effects of chronic TNFα treatment on the eNOS promoter. Finally, we have demonstrated that AS expression is increased by the PPARγ agonist drug, troglitazone, due to enhanced activity at a distal AS PPAR responsive element (PPRE). To expand our understanding of AS transcriptional regulation, we explored several pathways. First, we tested whether AS overexpression in endothelial cells enhances NO production. Our data demonstrates that AS overexpression
significantly increased basal NO production within 6 hours of transfection. Since insulin is a key regulator of vasodilation and vascular function, the hypothesis that insulin enhances AS expression and NO production was tested. Western blot and real time PCR experiments showed that AS protein and mRNA expression were up-regulated by physiological doses of insulin. Insulin coordinately regulated eNOS expression. Luciferase assay data also suggested that insulin may be activating a distal AS promoter element. Nitric oxide assays demonstrated that insulin alone can increase NO production and that it also acts synergistically with bradykinin and the calcium ionophore A23187 to increase NO production. This suggests that insulin up-regulation of AS is part of the mechanism by which it increases NO production. Since VEGF mediates important endothelium-specific functions such as angiogenesis and vasodilation, the hypothesis that VEGF enhances AS expression and NO production was examined. Western blot analysis demonstrated that VEGF increases AS expression. We also demonstrated a time-dependent increase in NO production in response to VEGF. Finally, we investigated the effects of ceramide on AS and eNOS expression. Ceramide is a bioactive sphingolipid with roles in cell signaling and apoptosis. Often times, the pathogenic effects of TNFα are mediated by ceramide. This prompted the hypothesis that ceramide would diminish AS expression under pathogenic conditions. Western blot analyses demonstrated that ceramide decreased AS and eNOS expression. In addition, ceramide diminished eNOS activation. Collectively, the data in this Chapter suggests that AS expression is highly regulated in a manner that is consistent with its role in supporting the catalytic activity of eNOS.
Materials and Methods

*Bovine Endothelial Cell Culture*: Bovine aortic endothelial cells (BAEC) were isolated by our laboratory from bovine aorta following the procedure of Gospodarowicz *et al* [1], and were used from passages 4-10. The study of bovine AS is supported by the extensive use of BAEC in research and the fact that AS shares a high sequence identity between human [2], bovine [3], rat [4] and mouse [5]. BAEC were cultured at 37°C under an atmosphere of 5% CO₂ in complete Dulbecco’s Modified Eagle’s Medium (DMEM) (1 g/L glucose, Mediatech) that contained 10% fetal bovine serum (Hyclone Laboratories), 100 units/ml penicillin and 100 μg/ml streptomycin (Mediatech). Cells were treated once they reached confluence.

*AS Expression Vector*: The AS plasmid contains both a V5 and 6X-His tag at its C-terminus. The vector map and sequence are below:
Figure 2: AS Expression Vector Map

-43bAS_pcDNA3.1V5HisB (updated 04Aug05)

6770 bps

BglII  NdeI  KpnI  BamHI  FseI  BsaBI  XhoI  BsrGI  EcoRI  EcoRV  BstXI  NotI  XhoI  XbaI  SacII  Pmel

AS Insert

AS start

V5

6xHis
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**Figure 3: AS Expression Vector Sequence.** Green = AS start; Red = AS stop; Turquoise = codons mutated to make T131, S180 and S189 variants in Chapter Two.
**Figure 3 (continued)**

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**AS Overexpression:** Experimental plasmids (2 μg/well of a 6 well dish) were transiently transfected into BAEC using either Lipofectamine 2000 (Invitrogen) or Fugene (Roche) in serum free Opti-MEM I (Invitrogen) as indicated. After 4 h, media
was replaced with Dulbecco’s modified Eagle’s medium containing 10% serum and cells were cultured for 6 or 24 h.

*Western Blot:* For western blots, equal amounts of protein (measured via BCA assay, Pierce) from clarified lysates were separated on polyacrylamide gels (BioRad), transferred onto polyvinylidene fluoride membranes (PVDF, Millipore) and blotted with indicated antibodies [AS, eNOS, phospho-eNOS (S1177) (BD Biosciences)]. Where appropriate, GAPDH (Novus Biologicals) was used as a loading control.

*RNA Isolation and Real Time PCR:* Total RNA was isolated using Tri Reagent following the manufacturer’s instructions (Sigma). RNA was treated with DNase (Ambion DNA-free). Two μg of RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit per the manufacturer’s instructions (Applied Biosystems). A 20 μl reaction was prepared with 10 μl 2X reaction mix (10X RT buffer, dNTP’s, 10X RT random primers, 10 X MultiScribe Reverse Transcriptase, RNase inhibitor and nuclease-free water) and 10 μl RNA. The following thermal cycler parameters were utilized for the reverse transcription: 25°C for 10 min, 37°C for 120 min, 85°C for 5 sec and hold at 4°C. Real time quantitative PCR was performed using the AS specific primers ASL200 and ASR352 utilizing a taq-man probe and with the eNOS specific primers eNOSL1075 and eNOSR1226 utilizing SYBR green as described previously by our laboratory [6, 7]. Results were normalized to GAPDH utilizing the primers GAPDHL351 and GAPDHR508. Primer sequences are below:
### Table 2: Primers Used for Real Time PCR.

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<td>GAPDHR508</td>
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**Luciferase Vector Construction:** For the luciferase vector, the 189 and 3075 base pair (bp) regions of the AS promoter were constructed as described previously [6, 8]. Briefly, luciferase reporter constructs were designed to include the AS promoter and 5′-UTR up to the AUG start codon cloned upstream of the luciferase gene. The left primers ASL189 (5′-GCACTCGAGATCTGCAGGTGGCTGTGAA) and ASL-3075 (5′-GTACCTCCACTGAAATTGAA) and were combined with ASRluc, (5′-ATAGAATGGCGCCGGGCGTTTCTTTATGTTTTTGGCGTCTTCCATCGTGACGT GACCAGCGGC) to amplify the AS promoter with an XhoI site on the 5′ end and an NcoI site on the 3′ end which were used to clone into the vector pGL3Basic (Promega). This strategy took advantage of an NcoI site within the luciferase gene, close to the start codon, to allow for the AS 5′-UTR to be cloned adjacent to the start codon.

**Luciferase Assays:** BAEC were cultured as described above and plated in a 24 well plate prior to transfection. Experimental plasmids (200 ng each) and renilla control plasmid pRL-TK (50 ng) were transiently transfected into BAEC using Transit-LT1 (Mirus) in serum free media. After 4 hours liposomes were removed and incubation continued for 24 hours. Cells were treated with insulin (10 nM, 2 hours). Cells were lysed
using passive lysis buffer (Promega). Ten μl lysate was assayed for luciferase and renilla activity using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions. Luciferase and renilla activity were measured as relative light units (RLUs) using a luminometer (Turner Designs). All results were normalized to renilla expression.

**AS Promoter Analysis:** The AS promoter elements that are identified in the discussion were found by doing a promoter search with 2 KB of the AS promoter. The promoter sequence for human AS mRNA with accession number NM_000050 was found utilizing the Transcriptional Regulatory Element Database (TRED) (http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home). The promoter sequence was then analyzed utilizing the Transcription Element Search System (TESS) database (http://www.cbil.upenn.edu/cgi-bin/tess/tess).

**Nitric Oxide Assays:** Nitric oxide released into tissue culture medium was measured utilizing the fluorescent probe 2,3-diaminonaphthalene (DAN) as described previously [9]. BAEC’s were serum starved overnight prior to all treatments except in the A23187 experiments. Briefly, at the indicated time points after treatment with insulin, A23187 (calcium ionophore), ionomycin (calcium ionophore), bradykinin or VEGF as indicated, aliquots of tissue culture medium were collected. Then, freshly prepared DAN reagent in 0.62 M HCl was added to culture supernatant, mixed immediately and incubated for 15 minutes. The reaction was stopped with a final concentration of 2.8 M NaOH and the samples were read on a BMG Fluostar Galaxy Spectrofluorometer.
exciting at 360 nm and emitting at 405 nm. Total protein was measured via the BCA assay, and data are presented as pmol nitrite produced per mg protein or as fold change over control.

To measure intracellular NO production in fixed cells, the NO-specific fluorescent dye, 4,5-diaminofluorescein diacetate (DAF-2 DA), was utilized as described by Montagnani et al [10]. Briefly, BAEC’s grown to confluence were loaded with DAF-2 DA (final concentration 1 µM, 20 min, 37 C) and then rinsed three times, kept in the dark, and maintained at 37 C. Cells were treated as indicated. Fluorescence intensity was imaged utilizing a Nikon Eclipse E1000 Fluorescence Microscope.

Statistical Analyses: Statistical analysis was conducted with a Student’s T-test of at least 3 independent experiments. Data is presented as the average +/- the standard error of the mean.

Results

AS Overexpression Enhances Endothelial NO Production: We hypothesized that since AS is an important mediator of the ability of eNOS to produce NO, its overexpression would enhance basal NO production. As seen in Figure 4A, transient overexpression of AS utilizing constant amounts of AS plasmid with increasing amounts of transfection reagent led to the generation of BAEC’s that expressed increasing amounts of AS. This led to enhanced production of NO that reached a plateau at the mid-
range of AS overexpression, likely due to the inability of eNOS to process the excess substrate without any additional stimulation of its activity. In a similar experiment, the empty vector was also transfected with constant plasmid DNA and increasing amounts of transfection reagent (Figure 4B). The results confirmed that it is the overexpression of AS per se that leads to the increase in NO production. These experiments were carried out after 24 hours of AS overexpression and significant cell loss was visually noted. To determine whether the cell loss was due to some condition associated with overexpression or to the enhanced NO production, the experiment was repeated but the NO measurement was conducted after only 6 hours of AS overexpression since no significant cell loss was noted at that time point. As shown in Figure 4C, NO production was enhanced at the 6 hour time point and this corresponded with a small but detectable overexpression of AS. This suggested that the cell loss was caused by the AS-mediated increase in NO production. Figure 4D demonstrates that AS overexpression does not have a consistent effect eNOS expression which suggests the effects on NO production are directly associated with the overexpression of AS.
Figure 4: AS Overexpression Enhances Nitric Oxide Production. (A) BAEC were serum starved overnight then transfected with 2 μg AS plasmid DNA with increasing amounts of Fugene transfection reagent (4, 5, 6, and 8 μl which correspond to AS0, AS1, AS2, AS3 and AS4, respectively). Graph demonstrates a nitric oxide assay with data presented as fold NO produced compared to control. Blot demonstrates expression levels of AS that correspond to the NO values. * p < 0.007 (n = 3). (B) Nitric oxide assay of BAEC overexpressing AS or an empty vector conducted as in A, except Fugene amounts were 3, 4, 5, 6, 7 and 8 μl and this time the empty vector was transfected with all conditions. (F3:D2 = 3 μl Fugene and 2 μg plasmid DNA, etc.). Data is presented as pmol nitrite/mg protein (n = 1). (C) NO assay in cells transiently transfected with the AS expression vector for 6 hours. This time, Lipofectamine 2000 was the transfection reagent used. Graph demonstrates a nitric oxide assay with data presented as fold NO produced as compared to control. Blot demonstrates expression levels of AS that correspond to the NO values (n = 2). (D) Representative blot demonstrating the lack of effect of AS expression on eNOS expression (n = 2).
NO Production in Transfected Cells

F3:D2, F4:D2, F5:D2, F6:D2, F7:D2, F8:D2

Fugene to DNA Ratio

pmol nitrite/mg protein

AS

EV

Overexpressed AS

Endogenous AS
AS Expression and Function are Enhanced by Insulin: Insulin is known to enhance NO production and is a key mediator of vascular health. Part of its atheroprotective properties stem from direct effects on eNOS expression. We hypothesized that AS expression would also be enhanced by insulin. To test this, time course experiments were carried out and it was determined that insulin enhances AS expression most consistently after 2-4 hours of treatment (Figure 5A). We then conducted dose response experiments to test the impact of 1-1000 nM insulin (2 hours) on AS and eNOS expression. Insulin enhanced AS and eNOS expression coordinately. The highest expression was seen at the 100 nM dose, but 10 nM still led to a significant increase in expression (Figure 5B & C). Additional experiments were conducted with the 2 hour time and 10 nM dose and the effects of insulin on AS expression were confirmed (Figure 5D & E).
Figure 5: Insulin Increases AS and eNOS Protein Expression. (A) BAEC were serum starved ON and cells were treated with insulin (100 nM) for the indicated times (n = 3). (B) BAEC were serum starved overnight then treated with insulin (1-1000 nM) for 2 hours. Representative western blot demonstrating expression of AS and eNOS. GAPDH was used as a loading control. Data is represented as fold change over control. (C). Densitometry of 2 independent experiments normalized to GAPDH. (D) Representative blot demonstrating expression of AS and eNOS in response to 10 nM insulin treatment for 2 hours. (E) Densitometry of C normalized to GAPDH demonstrating fold change over control (n = 5). * p < 0.004
C

![Graph showing relative expression of AS, eNOS, and GAPDH under control and insulin conditions.]

D

![Western blots showing protein expression of AS, eNOS, and GAPDH under control and insulin conditions.]

E

![Graph showing relative expression of AS and eNOS under control and insulin (10nM) conditions. Asterisks indicate significant differences.]
To determine whether the increase in AS protein expression was related to an increase in mRNA, real time PCR experiments were carried out. The data demonstrated that insulin also increases AS mRNA (Figure 6A). A similar trend was noted for insulin effects on eNOS mRNA. (Figure 6B).

**Figure 6: Insulin Increases AS and eNOS mRNA Expression.** BAEC were serum starved overnight then treated with insulin (10 nM) for 2 hours. (A) Real time PCR results demonstrating AS in control (gray bar) versus insulin treated cells (black bar). * p < 0.02 (n = 4). (B) Real time PCR demonstrating increase in eNOS expression with insulin treatment. (n = 2)
To determine whether insulin mediates the expression of AS by enhancing activity at specific AS promoter regions, we tested the effect of insulin on a proximal promoter construct (189 bp) versus a full length promoter construct (3075 bp). Luciferase assay data suggested that there may be distal AS promoter elements regulated by insulin (Figure 7).

Figure 7: Insulin Enhances AS Promoter Activity at a Distal Element. BAEC were serum starved overnight then treated with insulin (10 nM) for 2 hours. Luciferase assay results comparing 189 versus 3075 base pair promoter constructs. Gray bars represent vehicle treated cells and black bars represent insulin treated cells. Data is presented as relative luciferase units normalized to renilla expression. (n = 1)

Insulin is known to enhance NO production. To assess whether insulin was leading to an increase in NO production in our system and to correlate increased AS expression to a functional enhancement of the citrulline-NO cycle, we utilized two methods. First, we treated the cells with insulin, bradykinin, A23187 (calcium ionophore)
or a combination of treatments and measured NO released into the medium. Although this assay was not sensitive enough to reliably measure the effect of insulin alone, we were able to detect an increase with bradykinin that was enhanced by insulin (Figure 8A). Insulin also demonstrated the ability to enhance NO production above what the calcium ionophore A23187 can do alone (Figure 8B). We then utilized a probe that can measure intracellular NO levels in fixed cells. With this assay, we did see an increase in NO production with insulin treatment alone as evidenced by a visually determined increase in green fluorescence (Figure 8C). As expected, this increase was less than what was seen with the calcium ionophore ionomycin.
**Figure 8: Insulin Enhances Stimulated and Basal NO Production.** (A) BAEC were serum starved overnight then treated with insulin (100 nM), bradykinin (10 µM) or both for 4 hours. NO assay was conducted by measuring nitrite released into the medium before and after treatment (n = 3). (B) BAEC were treated with insulin (100 nM), A23187 (1 µM) or both for 4 hours. NO assay was conducted as in A (n = 1). (C) BAEC were serum starved overnight then loaded with 1.0 µM DAF2-DA for 20 minutes. Cells were then treated with insulin (100 nM) or ionomycin (2 µM) for 10 minutes. Cells were fixed and imaged utilizing fluorescent microscopy (ex 480 nM; em 510 nM).
**VEGF Regulates AS Expression and Enhances Endothelial NO Production:**

VEGF is an important mediator of endothelial function. We tested the hypothesis that part of the mechanism of VEGF-mediated vascular protection is associated with an increase in AS expression. First, we measured the time dependency of NO production in response to VEGF. As seen in Figure 9A, after 10 minutes of VEGF treatment, there was a statistically significant increase in NO production over untreated cells that continued to increase up to our final time point of 2 hours. Then, western blot analyses were conducted. The results demonstrated that VEGF increases AS and eNOS expression within 2 hours of treatment (Figure 9B). Importantly, as demonstrated and discussed further in Chapter Three, AS activity is required for maximum effects of VEGF on NO production (Figure 16, Page 122).
Figure 9: VEGF Increases Endothelial Nitric Oxide Production and Upregulates AS and eNOS Expression. (A) Nitric oxide assay measuring NO release into tissue culture medium (pmol nitrite/mg protein) by BAEC’s treated with VEGF (100 ng/ml) from 5 minutes to 2 hours. Diamonds represent control samples and squares represent VEGF treated samples. * p < 0.02; ** p < 0.0003 (n = 3). (B) Representative western blot of BAEC’s treated with VEGF (100 ng/ml) for 2 hours. Blot demonstrates expression of AS and eNOS. GAPDH was used as a loading control. (n = 2 for AS and n = 1 for eNOS)
Ceramide Diminishes AS and eNOS Expression and Suppresses eNOS Activation:

Chronic elevations in ceramide impair vascular function and diminish eNOS expression. Often times, ceramide mediates the pathogenic effects of TNFα on insulin signaling and other pathways. To test whether chronic ceramide elevations impact AS and eNOS expression, western blot analyses were conducted. When BAEC were treated with ceramide (10 µM) for 16 or 24 hours, AS and eNOS expression was diminished in a time dependent manner (Figure 10A). In addition, the increase in eNOS phosphorylation seen with insulin treatment was diminished when BAEC were pre-treated with ceramide (Figure 10B).
Figure 10: Ceramide Diminishes AS and eNOS Expression and Suppresses eNOS Signaling. (A) Representative western blot of BAEC's treated with ceramide (10 μM) for indicated times after overnight serum starvation. Blot demonstrates expression of AS and eNOS. GAPDH was used as a loading control (n = 1). (B) Representative blot of BAEC that were serum starved for 2.5 hours prior to pre-treatment with ceramide (cer; 10 μM) for 5 minutes followed by insulin treatment for 30 minutes (ins; 100 nM). Blot depicts eNOS phosphorylation (peNOS S1177) and total eNOS. GAPDH was used as a loading control (n = 1).
Discussion

In this Chapter, we made several important observations. First, we demonstrated that transient overexpression of AS leads to enhanced NO production. This increase was above the basal contribution of the endogenous citrulline-NO cycle. These findings are supported by the work of Xie et al. [11] which demonstrates that overexpression of AS in vascular smooth muscle cells (VSMC) increases NO production when cells are stimulated with lipopolysaccharide (LPS) and interferon gamma (IFNγ). Under those conditions, the effects they noted were related to inducible nitric oxide synthase (iNOS) and not eNOS. Even with stimulation, they saw about a 3-4 fold increase in NO production in stably transfected VSMC. In our case, we were monitoring basal effects and noted a 2 fold increase in NO production when AS was transiently overexpressed compared to untransfected or empty vector transfected controls. Our results implied that the effects we saw involved the AS-eNOS axis and not the AS-iNOS axis since a stimulant was not provided to induce iNOS. The fact that we saw the effects by 6 hours also suggested that iNOS is not involved since such a short time period of time without any stimulation would preclude iNOS induction. Further, the expression of eNOS was not consistently affected by AS overexpression nor did any small variations in eNOS expression correlate with the increases in NO production. This suggests the increase in NO production was a direct result of AS overexpression.

Furthermore, our studies support our previous work that demonstrates a significant loss of NO production and endothelial cell viability when AS is knocked
down with siRNA [12]. In those studies, reducing AS expression was directly linked to reduced expression of Bcl-2 and increased caspase activity. Thus, the cell loss was attributed to apoptosis. Further, it was demonstrated that apoptosis could be rescued with an NO donor. Therefore, apoptosis was mediated by the diminished production of NO as a direct consequence of decreased AS expression and activity. The work presented here alludes to the converse scenario. An increase in AS expression led to a significant increase in NO production, despite saturating levels of NO in the medium and without a change in eNOS activity or expression. In addition, the increase in NO production caused a significant cell loss, likely due to apoptosis. The fact that alterations of AS expression by overexpression or via treatment with various stimulants was associated with enhanced nitric oxide production also supports the body of evidence that demonstrates that AS is rate limiting for NO production. In addition, it is known that the recycling of arginine from citrulline is the preferred source of substrate for eNOS-mediated NO production, despite available transport systems and excess intracellular arginine levels [13, 14]. Therefore, our work also supports the mounting evidence that in endothelial cells, the citrulline-NO cycle is a tightly coupled system that generates a dedicated source of arginine for eNOS-mediated NO production [15, 16].

A second set of findings that are collectively important to note is that physiologic (insulin, VEGF) or pathogenic (ceramide) biological molecules affect AS expression coordinately with eNOS. This coordinate regulation is essential for the appropriate level of NO to be produced. Since AS is rate limiting for the production of NO by eNOS [11, 13, 14, 17], it is logical that its expression and function must be controlled in a manner
consistent with the substrate needs of eNOS. A possible exception to this would be in cases where NO levels have gone beyond physiological concentrations, which might lead to a decrease in AS function in order for NO production to be reduced. Although ultimately, this would lead to a coordinate downregulation of eNOS function, the initial response might be one of discoordinate regulation.

Several important biological treatments were explored in this Chapter and each one has important implications. We noted modest, yet significant increases in AS and eNOS expression in response to insulin. This work is supported by the work of Oyadomari et al. [18] who demonstrated that in rats with type 1 diabetes due to streptozotocin (STZ) treatment, AS and eNOS expression in whole aorta is enhanced initially and then decreases with longer duration of diabetes. The regulation is coordinate, much like what we see in cell culture models, and suggests the initial increase is a compensatory response while the end result is dysregulation of the citrulline-NO cycle. There is some controversy related to whether eNOS expression is diminished in animal models of diabetes or vascular disease [19]. This is likely due to differences in the animal models themselves and the treatment protocols utilized. In addition, mechanisms other than expression levels may underlie some forms of vascular disorders. Despite the controversy, it is clear that eNOS dysfunction is a hallmark of vascular disorders. Our work certainly demonstrates that AS is also a very important player and more research is needed to delineate the role of AS in diabetic animals and humans.
The role of insulin in regulating AS expression may be due to the direct effects of insulin in regulating the AS promoter. Although the results presented regarding enhanced AS promoter activity at a distal site in response to insulin are preliminary, there are several AS promoter elements that are known to be insulin responsive such as SP-1, USF and HIF1α [20-22]. Therefore, it is possible that there are several regions in the AS promoter that might regulate the insulin-mediated upregulation of AS mRNA and protein seen in our studies. In addition, we saw similar increases in eNOS expression and it has already been demonstrated that eNOS has at least two insulin responsive elements: SP-1 and AP-1 [23].

Our work suggests that the functional consequence of insulin up-regulation of AS expression is an increase in NO production. The increases we noted with insulin were generally modest and were most prominent when other stimulants, such as bradykinin, were used. This leads to our hypothesis that insulin is not necessarily a “stimulant” for NO production. Rather, we believe that the function of normal insulin signaling is to allow for NO stimulating pathways to be basally activated and ready for additional stimulation by vasodilators. Without insulin signaling, the optimal function of stimulants would be blunted. One key deficiency in our understanding of the upregulation of AS expression by insulin and the concomitant increase in NO production is whether these effects are direct or simply due to known effects on eNOS. To fill in this gap, future work is needed to determine whether insulin increases AS enzymatic activity.
Despite some of the gaps in our understanding of how insulin directly affects AS activity, our work with VEGF, another important mediator of vascular health with direct effects on the endothelium [24], supports the hypothesis that AS plays a direct role. Like insulin, we found that VEGF leads to a coordinate upregulation of AS and eNOS protein expression with a concomitant and significant increase in NO production. Our first hint that AS activity is directly involved comes from experiments that demonstrate that the maximal increase in VEGF-mediated NO production is blunted by a specific AS inhibitor. This data is presented and further discussed in Chapter Three, Figure 16, Page 122. Whether the effect of VEGF on AS protein expression is due to an increase on mRNA expression or stability is yet to be determined. However, there is some evidence in the literature that VEGF regulates the expression of some genes such as tissue factor and metallothionein via promoter activation [25]. It is possible there is a similar mechanism in place for AS. Another important finding from this work is the novel link between AS and angiogenic pathways. Although the function of AS in arginine regeneration has been associated with tumor survival and angiogenesis [26], a link between AS activity per se and angiogenic factors has not previously been described. Additionally, the AS-VEGF axis has not been defined in endothelium.

Our finding that chronic ceramide treatment diminishes AS and eNOS expression demonstrated that pathogenic environments lead to a dysfunction of the citrulline-NO cycle as a whole and not just eNOS. The effects of ceramide on AS expression have not been studied and most studies related to eNOS have determined that ceramide causes inhibition of eNOS activation, not expression. In fact, the only study that assessed eNOS
expression in response to ceramide in human endothelial cells found that although NO production was diminished due to elevations in ROS, eNOS expression was enhanced in an ineffective compensatory mechanism when treated for 10 or 16 hours [27]. In our study, both AS and eNOS expression were diminished in a time dependent manner at 16 or 24 hours when using the same dose used by the previous study [27]. The discordance of results may be due to the cell type used or the differences in ceramide preparations from different manufacturers. More likely, the issue is related to the fact that ceramide can have both beneficial and inhibitory effects [28] and this might explain the disparity. Overall, this implies that multiple mechanisms are disrupted in disease states that ultimately lead to the inability of the endothelium to produce adequate amounts of NO with downstream consequences such as atherosclerosis or myocardial infarctions.

The pathways we have studied related to the regulation of AS expression: TNFα, PPARγ agonists, insulin, VEGF and ceramide are relevant not just individually but also due to the fact that there is a great deal of cross talk between these pathways. One example is that ceramide mediates the pathogenic effects of chronic elevations of TNFα by inhibiting insulin actions [29]. Also, one mechanism by which PPAR agonists improve insulin sensitivity and improve NO function is by reducing the damaging effects of TNFα [30]. VEGF signaling, NO production and angiogenesis are all impaired in insulin resistant conditions [31-34]. These associations and the pivotal role of AS function in all these pathways improve our global understanding of vascular biology.
Another important observation about AS expression is that in tissues where its function is in the urea cycle, such as liver, expression is very high [35]. On the other hand, in tissues where AS functions with iNOS to produce NO (such as vascular smooth muscle and glial cells), its expression tends to be very low or even undetectable basally but is highly inducible by cytokines leading to iNOS induction and high output NO production [36-38]. Our findings suggest that in the endothelium, AS expression is basally repressed and this repression is removed in response to stimuli to increase NO output. This proposed de-repression mechanism leads to a modest change in AS expression in response to the multiple mechanisms we have explored up to this point (~2-3 fold). It turns out that eNOS transcriptional regulation in general also changes on the order of about 2-3 fold [39]. Although this might seem insignificant, the modest increases in NO associated with this change in expression can lead to pronounced changes in vascular tone. Since in the endothelium, eNOS functions to produce constant yet relatively low levels of NO, our model of modest changes in AS expression fits well with this functional paradigm.

Thus far, we have found an important pattern of coordinate regulation of AS and eNOS expression. It is important to note in this discussion that the studies led by Laura Pendleton in our lab have uncovered a unique translational regulatory mechanism that involves the expression of different lengths of AS message due to increasing lengths of the 5’ UTR [40]. The longer forms of message are endothelium specific and encode for a unique, small protein called Argininosuccinate Synthase Regulatory Protein (ARP). This protein regulates AS translation in trans since it suppresses the translation of the short
form of message that actually encodes for full length AS [7]. So far, this mechanism is unique to AS and seems to allow regulation of tissue-specific AS function. Thus, this is one example of regulation of AS that may be completely distinct from eNOS regulation. On the other hand, this may turn out to be a novel global paradigm that allows for the vast complexity of human biology despite a relatively small genome.

Overall, the studies presented in this Chapter further define the central role of AS for NO production and vascular health by uncovering several mechanisms that regulate its expression and lead to concomitant changes in endothelial NO production. These findings are significant since vascular disorders are sometimes characterized by reduced levels of eNOS expression [41-45]. Thus, we predict an important role of AS expression in health and disease.

References


CHAPTER TWO
ARGININOSUCCINATE SYNTHASE PHOSPHORYLATION

Overview

Argininosuccinate synthase (AS) is essential for endothelial nitric oxide (NO) production and its regulation in this capacity has been studied primarily at the transcriptional level. The dynamics of vascular function suggest that an acute regulation system may mediate AS function. This premise underlies our hypothesis that AS is phosphorylated in vascular endothelium. Since serine/threonine phosphorylation has been identified as the key mechanism mediating acute nitric oxide production, we focused on these modifications. We began our studies by conducting a bioinformatic analysis of the AS protein sequence utilizing 4 different databases. We identified 31 putative sites of AS serine/threonine phosphorylation that were positive hits in at least 2 of the 4 databases. Immunoprecipitation and immobilized metal affinity chromatography demonstrated that AS is an endogenous phosphoprotein. An in vitro kinase screen revealed that protein kinase A (PKA) and protein kinase c alpha (PKCa), kinases that enhance NO production via eNOS activation, phosphorylate AS. Vascular endothelial growth factor (VEGF) was identified as a candidate pathway for regulating AS phosphorylation since it activated PKA, PKCa and eNOS. In addition, α-methyl-DL-aspartic acid (MDLA), an AS
inhibitor, diminished maximal VEGF-mediated NO production. Immunoprecipitation studies suggested that VEGF enhances AS phosphorylation. We then focused our studies on identifying specific sites of AS phosphorylation utilizing proteomics. Thus far, we have been able to identify the following sites of AS serine/threonine phosphorylation: T131, S134, S180, S189, S328. Analysis of the AS 3-dimensional structure revealed important structure-function hypotheses related to the identified sites. Site directed mutagenesis of T131, S180 and S189 to generate phospho-null (alanine substitution) and phospho-mimetic (aspartic acid substitution) variants revealed a decrease and partial recovery of NO production compared to wild type, respectively. In silico modeling of phosphorylation sites utilizing the human AS crystal structure revealed that T131, S134 and S328 are the most accessible sites for modification by phosphorylation. Overall, our data demonstrates that regulation of AS by serine/threonine phosphorylation is an important mechanism for ensuring NO homeostasis. Our work suggests that targeting the kinases that lead to AS phosphorylation is one avenue to normalize AS function and offers an attractive therapeutic option for vascular disorders.

Materials and Methods

Bioinformatics: The following bioinformatic databases were utilized to identify putative AS phosphorylation sites and kinases: NetworKIN (http://networkin.info/search.php), Phospho-Motif (http://www.hprd.org/PhosphoMotif_finder), NetPhosK (http://www.cbs.dtu.dk/services/NetPhosK/) and Group Based Prediction System
Additionally, kinase motifs were identified by searching for specific motifs in the AS sequence utilizing Expasy Prosite (Scan Prosite Tool: http://www.expasy.ch/tools/scanprosite/). Multiple sequence alignments were conducted utilizing ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The 3-dimensional human AS crystal structure was generated utilizing ViewerLite software version 5.0 (Accelrys Corporation, San Diego, California) or iSee software from the Structural Genomics Consortium (http://www.sgc.ox.ac.uk/iSee/).

**Bovine Aortic Endothelial Cell Culture:** See Chapter One, Page 64

**In Vivo $^{32}$P Orthophosphate Labeling:** BAEC were serum starved for 4 hours in phosphate-free DMEM. Cells were transfected with V5-His tagged AS for 24 hours (see Chapter One, Page 64 for a full description of this vector) and then biosynthetically labeled with $[^{32}\text{Pi}]$ according to the procedure of Michel *et al* [1]. Labeled cells were treated with 10 μM bradykinin for 1 hour. Cell lysates were prepared and immunoprecipitations were carried out using antibodies directed against the V5-tag of overexpressed AS. Following the isolation of immune complexes with protein G-agarose (Santa Cruz), the complex was eluted by heating in SDS-PAGE sample buffer. The samples were fractionated via SDS-PAGE (4-20% gradient gels; Bio-Rad). After drying the gel, detection of phosphorylated AS was via Phosphor Imaging (GE Healthcare).

**Immunoprecipitation and Western Blot:** To determine whether AS is an endogenous phosphoprotein, immunoprecipitations (IP) were carried out utilizing a
phospho-serine/threonine antibody (BD Biosciences) and an AS antibody (Everest Biotech). To assess regulation of AS phosphorylation by VEGF, IP were carried out utilizing an AS antibody (Everest Biotech). After serum starvation overnight, complete medium with serum was replenished for 2 hours to restore maximal basal phosphorylation levels or cells were treated with VEGF (100 ng/ml) as indicated in figure legends. BAEC lysates were prepared from 150 mm dishes by adding 1 ml NP-40 buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% NP-40) plus protease and phosphatase inhibitors (Calbiochem and Pierce, respectively) and scraping. Lysates were centrifuged at 12,000 x g at 4°C for 10 minutes. Equal amount of total protein (measured by BCA assay, Pierce) from clarified lysates was incubated with 200 µl Dynabeads protein G (Invitrogen) per ml lysate at 4°C for 1 hour for pre-clearing. Supernatants were collected and incubated with corresponding antibodies at 4°C for 4 hours. The antibody-antigen complex was then incubated with 200 µl/ml Dynabeads Protein G for 1 hour at 4°C. IP’s with same species normal IgG antibodies (Santa Cruz) were conducted in parallel as negative controls. Whole cell lysate (WCL) was utilized as a positive control for antibody reactivity. For western blotting methods, refer to Chapter One, Page 68. Membranes were blotted with indicated antibodies [eNOS, phospho-eNOS (S1177) (BD Biosciences); PKA, phospho-PKA (T497), phospho-PKA substrate, phospho-PKC substrate, phospho-serine/threonine-phenylalanine (Cell Signaling Technologies); phospho-serine (Millipore)]. Where appropriate, GAPDH (Novus Biologicals) was used as a loading control.
Affinity Chromatography: Two different methods were utilized to separate phosphorylated and non-phosphorylated protein. First, the Phosphoprotein Enrichment Kit from Pierce was utilized. Procedures were conducted according to supplied instructions. In brief, cells were washed in HEPES buffered saline and lysed in included lysis buffer + 0.25% CHAPS and protease/phosphatase inhibitors (HALT Protease and Phosphatase inhibitor cocktails, Pierce). An equal amount of protein (measured with the Coomassie Plus Protein Assay; Pierce) diluted to 0.5 mg/ml was placed in supplied columns and incubated on a platform rocker for 30 minutes at 4°C. The flow through was collected and after washing, phosphoproteins were eluted with supplied elution buffer. Flow through and eluted fractions were concentrated using iCON Concentrators (Pierce). Total protein was measured and western blots were conducted as above. The presence of AS in the phosphoprotein fraction was monitored using a total AS antibody (BD Biosciences). To demonstrate effectiveness of separation and enrichment of phosphoproteins, membranes were probed with [phospho-Akt (S473) (Cell Signaling Technologies)]. Additionally, cytochrome C (Cell Signaling Technologies), a non-phosphorylated protein, was used as a negative control. For the λ-phosphatase experiments, once cell lysates were collected, cells were treated 700 μg of λ-phosphatase per 100 μg protein for 20 minutes at 37°C. The IMAC procedure then was continued as above.

The second method involved the Qiagen PhosphoProtein Purification Kit. Procedures were according to supplied directions. In brief, after collecting cells by trypsinization and washing cell pellet in HEPES buffered saline, cellular proteins were
extracted in lysis buffer containing 0.25% (w/v) CHAPS, protease/phosphatase inhibitors, and benzonase for 30 min at 4°C and centrifuged at 10,000g at 4°C for 30 min to remove insoluble material. Total protein was diluted to a concentration of 0.1 mg/ml in a total of 25 ml of lysis buffer and was applied to a lysis buffer-equilibrated PhosphoProtein purification column at room temperature. The column was washed with lysis buffer and the phosphoproteins were eluted with 2 ml of PhosphoProtein Elution Buffer. The yield of phosphorylated protein was determined by the BCA assay. The flow-through samples were passed through two additional columns to ensure complete removal of phosphoproteins from the sample. The eluted and flow-through fractions were then concentrated by ultrafiltration in a 10-kDa cutoff Amicon Ultra column (Millipore Corporation) and equal amount of protein from each sample was subjected to SDS-PAGE. The presence of AS in the phosphoprotein fraction was monitored using a total AS antibody (BD Biosciences). To demonstrate effectiveness of separation and enrichment of phosphoproteins, membranes were probed with phospho-eNOS (S1177) (BD Biosciences). α-tubulin was utilized as a loading control.

**Purification of Bovine Argininosuccinate Synthase:** Bovine AS (NP_776317) was subcloned into the pET-28(c)+ vector (Novagen) and expressed in *E. coli*. The protein was subsequently purified via the fused His tag by affinity chromatography utilizing Ni-NTA His-bind resin per the manufacturer’s instructions (Novagen). Successful purification was verified via SDS-PAGE. Purity was ~90% as determined by densitometry as shown below:
**In Vitro Kinase Screen:** Purified AS was utilized to run in vitro kinase reactions according to the manufacturer’s instructions (SignalChem, Vancouver, BC). Kinases were selected by conducting a bioinformatic search for putative AS kinases and then choosing a subset of those kinases for the screen based on their known role in regulating NO production. The following kinases were screened: AMP-activated protein kinase (AMPK; subunits A1/B1/G1 and A1/B1/G2), casein kinase II (CKII; subtype α1), glycogen synthase kinase 3 beta (GSK3β), protein kinase A (PKA- catalytic unit cα), protein kinase C alpha (PKCα) and protein kinase G (PKG- subtype 1). In brief, reactions contained the following components: active protein kinase, 10X reaction buffer, protein kinase activator, $^{33}$P-ATP and 5-10 µg recombinant, His-tag purified AS. A positive control to test for kinase activity was carried out with the appropriate peptide substrate and a blank reaction was carried out with all assay components except substrate. The assays were initiated by the addition of $^{33}$P-ATP and the reaction mixtures were incubated at 30°C for 45 minutes. The assays were terminated by spotting the reaction mixture onto a phosphocellulose P81 plate. After 3 15-minute washes in 1% phosphoric
acid, the radioactivity on the P81 plates was counted via scintillation counting. For imaging, *in vitro* kinase reactions were carried out essentially as above except they were terminated upon addition of SDS-PAGE sample buffer. Reactions were fractionated on 10% SDS-PAGE gels (BioRad) and exposed to film. For the dose response experiments, the reactions were carried out as above, with the exception that AS protein (substrate) concentration was varied from 2.5-12.5 µg. To determine if pre-phosphorylation of AS was required for CKII or GSK3β to phosphorylate AS, an in vitro phosphorylation reaction was carried out with AS and PKC using cold ATP followed by the in vitro reaction with CKII or GSK3β utilizing radiolabeled ATP.

*Tnitric Oxide Assays:* See Chapter 1, Page 70 for the methodology employed to measure NO released into the medium utilizing the DAN assay. For the VEGF experiments, cells were treated with VEGF +/- an AS inhibitor, α-methyl-DL-aspartic acid (MDLA; Sigma) as indicated. The experiments with wild type and mutant AS were carried out by overexpressing AS (see below).

*Generation of AS Variants and Transient Transfections:* The AS expression vector, fully described in Chapter One, Page 64, was utilized to mutate identified phosphorylation sites to determine their role in NO production utilizing procedures routinely conducted by our laboratory [2]. Briefly, phosphorylated residues were mutated to alanines to mimic a non-phosphorylated state and to aspartic acid to mimic a constitutively phosphorylated state following the “Quick Change” protocol utilizing Pfu Turbo DNA Polymerase (Stratagene). We compared the NO production of wild type
versus phospho-mutant AS in BAEC’s since we have demonstrated a consistent and reproducible increase in NO production when wild type AS is overexpressed in BAEC’s (See Chapter One, Figure 4, Page 73).

Primers were designed according to the guidelines from Strategene for the successful use of their “Quick Change” protocol. For the S180A/S189A, the S189A variant was used as the template and the S180A primers were used to introduce the additional mutation. All variants were verified by sequencing. The following primers were utilized:

<table>
<thead>
<tr>
<th>Variant</th>
<th>Wild Type Sequence</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>T131A</td>
<td>C CGG TTT GAG CTC ACC TGC TAC TCG CTG G</td>
<td>C CGG TTT GAG CTC GCC TGC TAC TCG CTG G</td>
<td>C CAG CGA GTA GCA GCC GAG CTC AAA CCG G</td>
</tr>
<tr>
<td>T131D</td>
<td>C CGG TTT GAG CTC GCC TGC TAC TCG CTG G</td>
<td>C CGG TTT GAG CTC GAC TGC TAC TCG CTG G</td>
<td>C CAG CGA GTA GCA GTC CAG CTC AAA CCG G</td>
</tr>
<tr>
<td>S180A</td>
<td>CAA GAA CCC GTG GAG CAT GGA CGA GAA CCT G</td>
<td>CAA GAA CCC GTG GGC CAT GGA CGA GAA CCT G</td>
<td>C AGG TTC TCG TTC ATG GCC CAC GGG TTC TTG</td>
</tr>
<tr>
<td>S180D</td>
<td>CAA GAA CCC GTG GAG CAT GGA CGA GAA CCT G</td>
<td>CAA GAA CCC GTG GGC CAT GGA CGA GAA CCT G</td>
<td>C AGG TTC TCG TTC ATG TTC CAC GGG TTC TTG</td>
</tr>
<tr>
<td>S189A</td>
<td>AGA ACC TGA TGC ATA TC A GCT ACG AGG CTG GAA TCC</td>
<td>AGA ACC TGA TGC ATA TC C GCT ACG AGG CTG GAA TCC</td>
<td>AGA ACC TGA TGC ATA TC G ACT ACG AGG CTG GAA TCC</td>
</tr>
<tr>
<td>S189D</td>
<td>GGA TTC CAG CCT CTG AGG CTA TAT GCA TCA GGT TCT</td>
<td>GGA TTC CAG CCT CTG AGG CTA TAT GCA TCA GGT TCT</td>
<td></td>
</tr>
</tbody>
</table>

Experimental plasmids were transiently transfected into BAEC using Lipofectamine 2000 (Invitrogen) in serum free Opti-MEM I (Invitrogen). Two µg AS plasmid DNA was used per well of a 6-well dish or 10 µg per 100 mm dish. After 4 h, the
medium was replaced with Dulbecco’s modified Eagle’s medium containing 10% serum and cells were cultured for 24 h.

*Purification of Overexpressed AS:* Two methods were utilized to partially purify the AS expression vector. In order to obtain sufficient material for mass spectrometry analysis, at least 3 100-mm dishes were transfected per treatment group as described above and lysates were pooled. Cells were treated with either 20 or 100 nM okadaic acid, a serine/threonine phosphatase inhibitor, in an attempt to enhance phosphorylation signal for the experiments to characterize the AS expression vector.

The first method involved immunoprecipitation of overexpressed AS with a V5 antibody. Lysates were prepared utilizing NP-40 lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% NP-40) plus protease and phosphatase inhibitors (Calbiochem and Pierce, respectively) and scraping. Clarified lysates were pre-cleared with a protein G bead slurry (Protein G Plus Agarose, Santa Cruz) for 1 hour. Immunoprecipitation was conducted with an antibody against the V5 tag of the AS expression construct (Invitrogen) by incubating the pre-cleared lysate with antibody for 2 hours at 4°C. The protein G bead slurry was added to samples and incubated overnight at 4°C. Purified AS and associated proteins were eluted with 2X SDS-PAGE sample buffer.

The second method involved purification via the 6X-his tag of the AS expression vector. Magnetic Ni-NTA agarose beads (Qiagen) were utilized according to the manufacturer’s instructions. Briefly, cells were lysed in Buffer B-Tween + 1% NP-40
(100 mM NaH₂PO₄, 10 mM Tris·Cl, 8 M urea and 0.05% tween, pH 8.0). Lysates were cleared by centrifugation. Ni-NTA agarose beads were added (20 μl/ml) to the lysates and incubated with end-over-end rotation for 2 hours at 4°C. Cells were washed in Buffer C-Tween (same as buffer B, pH 6.3) and eluted with Buffer E-Tween (same as buffer B, pH 4.5).

To monitor expression levels, western blots were conducted as described previously (purified samples and whole cell lysates were compared) and membranes were probed with an AS antibody. To assess whether the purified vector was phosphorylated, membranes were probed with a phospho-serine antibody (Zymed). To visualize effectiveness of purification, duplicate gels were run, coomassie stained, dried by vacuum and photographed.

**Liquid Chromatography and Tandem Mass Spectrometry:** AS was transiently overexpressed in BAEC’s. Twenty four hours after transfection, cells were deprived of serum overnight. Cells were treated with insulin, bradykinin, insulin + bradykinin and okadaic acid. Overexpressed AS was purified by its fused 6X-His tag using Ni-NTA agarose magnetic beads (Qiagen) as described above. Proteins were separated by SDS-PAGE to identify the AS band (51 kD). A duplicate gel was run to confirm expression of the AS plasmid by western blot. Gel bands of interest were excised and destained. The protein disulfides were reduced with triscarboxyethylphosphine and then the cysteines were alkylated with iodoacetamide. In-gel trypsin digestion was used for proteolysis. The resulting peptides were extracted and concentrated prior to liquid chromatography...
coupled to tandem mass spectrometry (LC-MS/MS) analysis. Nanoflow reverse phase liquid chromatography was used to separate the peptides by hydrophobicity (LC Packings, Dionex, Sunnyvale, CA). Online detection was accomplished with an electrospray linear ion trap mass spectrometer (LTQ, Thermo, San Jose, CA). Peptide molecular weight measurements preceded ion selection, fragmentation, and fragment ion detection in MS/MS. Tandem mass spectra were assigned to peptide sequences using Mascot and Sequest database search algorithms. Sequence assignments were validated by manual inspection of the data.

In-Silico Modeling of AS Three Dimensional Structure: In silico models of AS with identified phosphorylation sites were generated by the University of South Florida Department of Chemistry by Dr. Wayne Guida and Daniel Santiago utilizing a Molecular Dynamics approach. Modeling was dependent on the original X-ray structure (PDB ID: 2NZ2). Substrate binding affinity was measured when sites were not phosphorylated versus phosphorylated (comparative docking). The methodology is composed of four phases:

I. Substrates were docked to original enzyme structure.

II. Phosphorylation models were created for each possible serine/threonine residue.

III. Low energy conformer for each phosphorylation model was identified.

IV. Substrates were docked into phosphorylation models and docking scores were compared.

Statistical Analyses: See Chapter One, Page 71.
Results

AS is an Endogenous Phosphoprotein: Since serine/threonine phosphorylation is a prominent mechanism that regulates acute eNOS function, we focused on these modifications. To assess whether endogenous AS is phosphorylated, several approaches were taken. First, a bioinformatic search for AS serine/threonine phosphorylation utilizing four different databases was conducted. The benefit of utilizing multiple databases is that they each utilize slightly different approaches. For example, NetPhos 2.0 utilizes trained neural networks for predictions [3]. On the other hand, NetworKin predicts \textit{in vivo} kinase-substrate relationships by augmenting the information gained from kinase substrate motifs with context for kinases and phosphoproteins [4]. Phospho-Motif predictions are based on known consensus kinase motifs as well as additional motifs that are curated from the literature [5]. Finally, Group Based Prediction System improves on the methods of standard prediction systems by grouping kinases into hierarchical structures and developing and approach to minimize false positives [6]. By cross referencing results from the 4 databases, the number of false positives should be reduced. Through these \textit{in silico} experiments, 31 sites were identified that were positive hits in at least 2 of the 4 databases (Table 4).
Table 4: Predicted AS serine/threonine phosphorylation sites. Data was generated by cross-referencing results from 4 different databases and choosing only sites that were positive hits in at least 2 of 4 databases.

<table>
<thead>
<tr>
<th>Position</th>
<th>Peptide</th>
<th>Position</th>
<th>Peptide</th>
<th>Position</th>
<th>Peptide</th>
</tr>
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<tbody>
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<td>2</td>
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<td>NP-WSMDE</td>
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<td>APNS-PDM</td>
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<td>GDGTTHS</td>
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<tr>
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<td>DGTHS</td>
<td>376</td>
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<td>115</td>
<td>KYV-SHGA</td>
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<td>THS-TALE</td>
<td>396</td>
<td>NIN-SLRL</td>
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<td>GMK-SRG1</td>
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<td>NKVTAK-</td>
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<td>PAG-TLY</td>
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</tbody>
</table>

To evaluate whether the \textit{in silico} identification of AS phosphorylation was occurring endogenously, immunoprecipitation of BAEC lysates was carried out with a phospho-serine/threonine antibody followed by immunoblotting with an AS antibody. As seen in Figure 12, AS co-immunoprecipitates when cellular proteins that are phosphorylated at serine and/or threonine residues are enriched. Similar results were obtained with the reverse immunoprecipitation, but in this case, the best results were obtained when probing with a phospho-serine and a phospho-serine/threonine/tyrosine antibody (Figure 12). In addition, in vivo $^{32}$P orthophosphate labeling was utilized to determine if AS is phosphorylated. AS was overexpressed, labeled with $^{32}$P then treated with bradykinin. Lysates were prepared and overexpressed AS was purified via immunoprecipitation. As shown in Figure 12, AS is phosphorylated basally and this increased upon bradykinin treatment.
Figure 12: AS is an Endogenous Phosphoprotein, Part I. (A) Representative blot (n = 3) of immunoprecipitation of BAEC lysates utilizing a phospho-serine/threonine antibody (IP pS/T) or a same-species normal IgG (IP IgG). The membrane was probed with an AS antibody (IB AS). WCL; whole cell lysate. (B) Representative blot (n = 3) of immunoprecipitation of BAEC lysates utilizing an AS antibody (IP AS) or a same-species normal IgG (IP IgG). The membrane was probed with a phospho-serine or a phospho-serine/threonine/tyrosine antibody (IB pS and IB pS/T/Y, respectively); pAS = phospho-AS. (C) Phosphor-image demonstrating the level of $^{32}$P incorporation in response to bradykinin stimulation (10µM) in BAEC’s where either AS (AS-V5-His) or an empty vector were overexpressed and immunoprecipitated (n = 1).

To further validate that AS is indeed an endogenous phosphoprotein in the endothelium, we utilized immobilized metal affinity chromatography (IMAC) to separate and enrich phosphorylated protein from total protein. This was done with two different
methods since the different resins utilized to bind phosphorylated proteins have affinity for some phosphoproteins and not others [7]. With the first method, the western blot analysis in Figure 13A demonstrated that a fraction of AS eluted from the column as a phosphorylated protein. As a control, membranes were re-probed with a phospho-Akt antibody. Phospho-Akt was observed in the eluted phosphoprotein fraction with a relative absence in the flow-through fraction (Figure 13A). As a negative control, membranes were re-probed with a cytochrome c antibody (cytochrome c is not phosphorylated) and no cytochrome c was observed in the eluted phosphoprotein fraction (Figure 13A).

Figure 13B demonstrates the similar results that were obtained with the second method. A fraction of AS eluted as a phospho-protein. This time, phospho-eNOS was utilized as a positive control to demonstrate the effectiveness of the separation of phosphoprotein from total protein (Figure 13B). In addition, these experiments were conducted with various treatments in an attempt to monitor changes in AS phosphorylation. There were no obvious changes in the amount of AS eluted in the phosphoprotein fraction. Because of the high affinity of these resins for phosphorylated proteins, it is nearly impossible to detect changes in binding to the column with a total protein antibody since a treatment may cause a change in phosphorylation sites rather than a net increase or decrease in phosphorylation. It became evident that this technique could not be used to monitor changes in phosphorylated AS without having a phospho-AS antibody. This was demonstrated in subsequent experiments where dephosphorylation reactions were carried out with λ-phosphatase prior to the IMAC experiments. While decreases in phosphorylation were noted when using a site specific phospho-Akt
antibody, there was little to no change in the amount of AS eluted when a total AS antibody was used. In order for these experiments to have worked, AS needed to be completely dephosphorylated prior to IMAC in order to see a significant change.

Finally, to make sure that the IMAC data was not an artifact, IMAC was conducted and then AS was immunoprecipitated from the eluted phosphoprotein fraction. As shown in Figure 13C, AS was successfully immunoprecipitated from the eluted fraction suggesting that the signal noted with IMAC is indeed a fraction of AS that elutes as a phosphoprotein and not simply a non-specific band at the AS molecular weight. Taken together, the immunoprecipitation, $^{32}$P labeling and phosphoprotein affinity chromatography strongly suggest that AS is an endogenous phosphoprotein in vascular endothelium.
Figure 13: AS is an Endogenous Phosphoprotein, Part II. (A) Representative blot (n = 3) demonstrating separation of phosphorylated protein from total protein utilizing immobilized metal affinity chromatography (IMAC). The top panel shows the membrane was probed with AS. Phospho-Akt was utilized as a positive control and cytochrome c was used as a negative control. (B) Representative blot of IMAC conducted as in (A) but with a different resin (n = 3). The blot shows the level of AS, phospho-eNOS or α-tubulin in the eluted (E) or flow through (FT) fractions in response to treatment with insulin (100 nM), okadaic acid (50 nM; OA) or H89 (n = 3). (C) Representative blot of the eluted phospho protein fraction of BAEC’s treated without (vehicle) or with λ-phosphatase (λ-pptase) prior to IMAC conducted as in (A) (n = 2). (D) Representative blot demonstrating IMAC conducted as in (B). The data represents AS expression in the whole cell lysate (WCL), the eluted IMAC fraction (IMAC) or from the IMAC fraction after immunoprecipitation with an AS antibody (IMAC + IP) in cells treated with okadaic acid (OA) at the doses indicated (n = 1).
Biological Relevance of AS Phosphorylation: In order to establish the biological significance of AS phosphorylation, we conducted a targeted in vitro kinase screen. First, we identified putative AS kinases utilizing four bioinformatic databases. From that list of kinases, the following were selected for the screen due to their known role in nitric oxide biology: AMP-activated protein kinase (AMPK), casein kinase II (CKII), glycogen synthase kinase 3 beta (GSK3β), protein kinase A (PKA) and protein kinase C alpha (PKCa). Akt1 was used as a negative control due to the lack of the consensus Akt phosphorylation motif in the AS protein sequence (R-X-R-X-X-S/T). The kinase screens were carried out with purified recombinant bovine AS and as shown in Figure 14A, PKCa and PKA were identified as AS kinases due to their ability to phosphorylate AS in vitro. Although there was an increase in counts above the blank reaction with AMPK and Akt1, this was below the threshold considered significant (Figure 14A). SDS-PAGE fractionation of in vitro kinase reaction products and visualization by film exposure confirmed that PKCa and PKA, but not Akt1, phosphorylate AS (Figure 14B). Repeat in vitro kinase reactions for GSK3β and CKII were carried out, but AS was pre-
phosphorylated with PKCa. CKII and GSK still were unable to phosphorylate AS in vitro (Figure 14C).

Figure 14: AS is Phosphorylated by PKCa and PKA. (A) In vitro kinase assays. Gray bars represent the incubation of purified AS minus kinase (Blank). Black bars represent the incubation of purified AS with the indicated kinase (Kinase). Kinase activity is represented as counts per minute (CPM). * p < 0.0002; ** p < 0.00003; (n = 3 for PKC, PKA and Akt; n = 1 or 2 for remaining kinases). (B) Representative image of in vitro kinase reactions as described in (A) but only showing PKCa, PKA and Akt. pAS; phosphorylated AS (n = 2). (C) AS was pre-phosphorylated with PKC (using cold ATP) then in vitro kinase reactions were carried out with GSK3β and CKII as in (A) (n = 1).
To test the dose-dependence of PKCα and PKA phosphorylation of AS, *in vitro* kinase reactions were conducted with a constant level of PKCα or PKA and varying AS concentrations (2.5 µg to 12.5 µg). The results demonstrate dose-dependent phosphorylation of AS by PKCα that plateaus at 5 µg of AS (Figure 15A). With PKA, the level of phosphorylation was linear up to 7.5 µg AS (Figure 15B). In addition, there are numerous PKA and PKC motifs in the AS protein sequence, and this supports the *in vitro* data generated (Table 5).
Figure 15: Dose Dependence of AS Phosphorylation. (A) In vitro kinase reactions carried out with a constant concentration of PKCα and increasing concentrations of AS (2.5-12.5 μg). Activity is represented as corrected CPM after subtracting blank (n = 1). (B) In vitro kinase reactions carried out with a constant concentration of PKA and increasing concentrations of AS (2.5-12.5 μg). Activity is represented as corrected CPM after subtracting blank (n = 3).
Table 5: Possible Sites Phosphorylated by PKA or PKCα. Data generated with bioinformatics databases. Only sites that were positive hits in at least 2 of 4 databases are shown.

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Possible Sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKA</td>
<td>S2, S6, S115, T119, T174, S180, S219, S278, S352, S365, S396, T410</td>
</tr>
<tr>
<td>PKCα</td>
<td>S2, S6, T91, S115, T119, T174, T208, S219, T242, S352, S365, S396, T410</td>
</tr>
</tbody>
</table>

To further support the biological significance of AS phosphorylation, we considered several candidate pathways that could regulate AS phosphorylation. Insulin, VEGF and bradykinin pathways were all tested. Preliminary data looked positive for all three pathways since all demonstrated the ability to enhance AS phosphorylation. The data was strongest with vascular endothelial growth factor (VEGF). Since both PKA and PKCα have been linked to VEGF and NO production, this pathway was investigated further. In Chapter One, Figure 9A, we demonstrated that VEGF significantly enhances NO production starting at 10 minutes and continuing up to 2 hours. In order to assess whether PKA, PKCα and eNOS were activated during acute stimulation of NO production by VEGF, we measured their phosphorylation after treatment with VEGF for 10 minutes. Indeed, PKA, PKCα and eNOS had increased levels of phosphorylation in response to VEGF, suggesting that these are key events that contributed to the enhanced production of NO (Figure 16A).
Although eNOS is known to be activated during VEGF stimulation to enhance NO production, it is not known whether AS activity is also enhanced and necessary for increased NO production during VEGF stimulation. To study this, the 2 hour time point was used in order to clearly distinguish VEGF-stimulated NO production under AS-inhibited and uninhibited conditions. BAEC’s were pre-treated for 1 hour with the AS inhibitor α-methyl-DL-aspartic acid (MDLA, 10 mM), an aspartic acid analogue that competitively inhibits AS [8]. The cells were then treated with VEGF (100 ng/ml) for 2 hours. As shown in Figure 16B, the VEGF-mediated increase in NO production was substantially diminished when endothelial cells were pre-treated with MDLA. When this experiment was repeated with a 10 minute treatment with VEGF, the AS inhibitor MDLA essentially abolished both basal and VEGF-stimulated NO production (Figure 16C).

To determine whether the requirement for AS activity for maximal VEGF-mediated NO production was related to AS phosphorylation, BAEC’s were treated with 100 ng/ml VEGF for 10 minutes. Lysates were prepared and immunoprecipitations were carried out utilizing an AS antibody. Subsequent western blotting with phospho-serine/threonine, phospho-PKC substrate and a phospho-PKA substrate antibody revealed that AS phosphorylation was enhanced by VEGF treatment (Figure 16D).
Figure 16: VEGF is a Candidate Pathway for Regulating AS Phosphorylation. (A) Representative western blot (n = 3) of BAEC treated with VEGF (100 ng/ml, 10 minutes). Membranes were immunoblotted with phospho-PKCa (pPKCa), total PKCa, phospho-PKA (pPKA), total PKA, phospho eNOS (peNOS), total eNOS and GAPDH (GAPDH). (B) NO assay of cells treated with MDLA alone (pre-treated for 1 hour), VEGF alone (2 hours) or VEGF + MDLA. *** p < 0.00005; * p < 0.05; n = 3 (C) Experiment conducted as in (B) with the exception that treatment with VEGF was for 10 minutes (n = 1). (D) Immunoprecipitation of cell lysates that were treated +/- VEGF (100 ng/ml, 10 min) with an AS antibody (IP AS) or a same-species normal IgG (IP IgG) followed by probing with phospho-serine/threonine (pS/T), phospho-PKC substrate (pPKC Substrate) and phospho-PKA substrate (pPKA Substrate) antibodies. IgG = non-specific IgG band; pAS = phosphorylated AS band (n = 3).
Identification of Specific Sites of AS Phosphorylation: In order to assess the best methodology to pursue mass spectrometry identification of AS phosphorylation, two approaches were taken to purify sufficient amounts of AS. For these experiments, we decided to utilize overexpression. Although this scenario is somewhat artificial, the low stoichiometry of phosphorylation prompted us to take measures to maximize the possibility of detecting phosphorylated-AS peptides. The first purification method involved immunoprecipitating overexpressed AS utilizing a V5 antibody to pull it down via its V5 tag. The expression levels of AS with this method were excellent and overexpressed AS was sufficiently purified (Figure 17A and B, respectively). In addition, AS, but not the empty vector control demonstrated phosphorylation at serine residues both basally and in response to okadaic acid treatment (a phosphatase inhibitor). There was no apparent increase in phosphorylation with treatment (Figure 17A). One possible problem with this methodology involves the fact that the heavy chain IgG (~55 kD) is inevitably pulled down (Figure 17B). Since it is close to the overexpressed AS molecular weight (~51 kD), IgG might interfere with the identification of AS.
Figure 17: Overexpression and Purification of AS Utilizing Immunoprecipitation. (A) Immunoprecipitation was utilized to purify overexpressed AS-V5-His utilizing a V5 antibody. Blot compares samples that were treated with increasing concentrations of okadaic acid (OA) as indicated. Left side of blot shows immunoprecipitated samples (IP V5) and right side shows whole cell lysate (WCL) of samples expressing AS (AS-V5-His) or an empty vector. Top blot was probed with AS antibody and both overexpressed (AS: OE) and endogenous AS (AS: E) can be detected. Bottom blot was probed with a phospho-serine (pS) antibody. (B) Image of duplicate gel that was coomassie stained. Red box indicates AS (51 kD). Black box indicates non-specific heavy chain IgG band (~50-55 kD). Protein marker is indicated by arrow.
This prompted us to test a second method that involved utilizing Ni-NTA magnetic beads to affinity purify overexpressed AS via its 6X- His tag. This method allowed for excellent enrichment and purification of the overexpressed AS (Figure 18A and B). It also allowed for a significant amount of endogenous AS to co-purify (Figure 18A). Considering the effectiveness of this method and the lack of IgG interference, it was chosen for downstream mass spectrometry experiments. In addition, as shown in Chapter One, Figure 4, AS overexpression leads to enhanced NO production, suggesting that overexpressed AS functions similarly to the endogenous enzyme. Overall, this data supports the use of the overexpression system to identify specific sites of AS phosphorylation.
Figure 18: Overexpression and Purification of AS Utilizing Ni-NTA. (A) Ni-NTA agarose was utilized to purify overexpressed AS-V5-His. Blot compares samples that were treated with increasing concentrations of okadaic acid (OA) as indicated. Left side of blot shows purified samples (Ni-NTA Purified) and right side shows whole cell lysate (WCL) of samples expressing AS (AS-V5-His) or an empty vector. Top blot was probed with AS antibody and both overexpressed (AS: OE) and endogenous AS (AS: E) can be detected. Bottom blot was probed with a phospho-serine (pS) antibody. (B) Image of duplicate gel that was coomassie stained. Red box indicates AS (51 kD). Protein markers are indicated by arrows.
In order to identify specific sites of AS phosphorylation, AS was overexpressed and cells were treated with okadaic acid, insulin, bradykinin, and insulin + bradykinin. AS was purified utilizing Ni-NTA agarose magnetic beads and cellular protein was separated utilizing SDS-PAGE. The band corresponding to overexpressed AS was excised and prepared for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. We have thus far identified the following sites of AS phosphorylation: T131, Y133, S134, S180, S189, Y207 and S328. The sites that have been identified on several occasions are T131, S180 and S189 while the remaining sites have not yet been confirmed. Assessment of the cumulative data did not provide clear determination as to whether any particular treatment enhanced phosphorylation at a specific site. We did note that the patterns of AS phosphorylation are indeed dynamic. Attempts were made to relatively quantitate the phosphorylation of AS +/- different treatments utilizing data from the liquid chromatography phase to enhance peptide identification via mass spectrometry. This data was also inconclusive. However, there were trends noted that suggested changes in phosphorylation patterns. In addition, the peptide 131-TCYS-134 contains multiple possible modifications. Their close proximity makes it impossible to determine which site is actually modified (i.e. T131 vs. S134). Figure 19 is an example of the data generated via LC-MS/MS. The spectrum at the top shows that under control conditions, T131 is not phosphorylated. The spectrum below it indicated that upon okadaic acid treatment, T131 becomes phosphorylated. The mass to charge ratio (m/z) difference of a peptide phosphorylated by a serine or threonine modification is ~80. The m/z difference of 11 between the non-phosphorylated vs. phosphorylated T131 was derived by the fact that the non-phosphorylated peptide was alkylated (m/z = 57). The
m/z difference between the non-phosphorylated and phosphorylated peptide with a charge of +2 was calculated as follows: \((80-57)/2 = 11\).

Figure 19: Identification of AS Phosphorylation Sites Utilizing Liquid Chromatography-Tandem Mass Spectrometry. AS was overexpressed and cells were treated with okadaic acid (100 nM) for 30 min. After purification and SDS-PAGE, the band corresponding to overexpressed AS was excised digested, and subjected to liquid chromatography-tandem mass spectrometry. Figure shows mass spectra of control (top) and okadaic acid (bottom) treated samples. In the control samples, T131 is not phosphorylated while in the okadaic acid samples, T131 is phosphorylated.
Although AS is the focus of this work, we did identify some novel eNOS phosphorylation sites that are worth noting. These experiments were carried out by immunoprecipitating eNOS as described in Chapter Three, Page 166, and subjecting the eNOS band to LC-MS/MS to identify phosphorylation sites. The novel sites of bovine eNOS phosphorylation were: T60, T62, T389, and S485.

Since the focus of this work is serine/threonine phosphorylation, further investigation into biological significance was conducted for only those modifications. First, to determine the evolutionary relevance of the sites, a multiple sequence alignment was conducted. As seen in Figure 20, T131, S134, S180, S189 and S328 are 100% conserved among mammals with the exception that humans have a serine at position 131 while other mammals have a threonine. The sites that were completely conserved even down to insects and *E. coli* were S180 and S189. The only minor substitution was a threonine at position 189 in *E. coli*. Overall, the mammalian AS sequences depicted in the figure are about 95% identical. Sequence identity then declines with the lowest being 25% for the *E. coli* sequence compared to human or bovine (per ClustalW analysis). With such a low level of conservation among some of the non-mammalian species, the persistence of phosphorylatable residues at some of the positions strongly suggests biological importance.
Figure 20: Multiple sequence alignment of AS Phosphorylation Sites. Panels show the peptide containing T131 and S134 (A), S180 and S189 (B) or S328 (C). Sites are highlighted in blue.
To further elucidate the biological role of these sites, an *in silico* experiment was conducted to identify putative kinases. As seen in Table 6, several important kinases were identified as possible kinases for the identified sites including PKA, AMPK, CKII and GSK3β. Interestingly, PKC motifs were not found at any of the AS phosphorylation sites identified so far.

**Table 6: Putative Kinases for Identified AS Serine/Threonine Phosphorylation Sites.**

Data was generated based on motif searches with Prosite or via bioinformatic databases.

<table>
<thead>
<tr>
<th>RESIDUE</th>
<th>PUTATIVE KINASES</th>
</tr>
</thead>
<tbody>
<tr>
<td>T131</td>
<td>AMPK</td>
</tr>
<tr>
<td></td>
<td>CKI &amp; CKII</td>
</tr>
<tr>
<td></td>
<td>MAPK</td>
</tr>
<tr>
<td>S134</td>
<td>CKI</td>
</tr>
<tr>
<td>S180</td>
<td>PKA</td>
</tr>
<tr>
<td></td>
<td>CKII</td>
</tr>
<tr>
<td></td>
<td>PKG</td>
</tr>
<tr>
<td>S189</td>
<td>CKII</td>
</tr>
<tr>
<td></td>
<td>MAPK</td>
</tr>
<tr>
<td>S328</td>
<td>GSK3</td>
</tr>
<tr>
<td></td>
<td>CKII</td>
</tr>
<tr>
<td></td>
<td>MAPK</td>
</tr>
</tbody>
</table>

Another methodology to explore biological significance involves utilizing the 3-dimensional structure of an enzyme to assess possible structure-function relationships. As indicated in Table 7, each of the identified AS serine/threonine phosphorylation sites is linked to physiologically relevant pathways. T131 and S134 are located in the N-terminal, nucleotide binding domain of AS, which has been shown to be involved in conformational change during catalysis in the *E. coli* enzyme. T131/S134 are also
adjacent to cysteine 132, a site that is reversibly nitrosylated in vascular smooth muscle and functions as a negative regulator of AS enzyme activity under conditions of excessive NO production. S180 is in the N-terminal domain but is part of the catalytic cleft. It is hypothesized to interact with ATP {per e-mail conversation with Dr. Jonas Uppenberg [9]}. Interestingly, S180 is mutated in citrullinemia (S180 → N), a disease characterized by diminished AS function. S189 is also in the N-terminal domain as part of the catalytic cleft and hydrogen bonds with citrulline. S328 is located in the catalytic domain. Additionally, it is adjacent to the AS caveolin-1 binding motif (discussed in Chapter Three), suggesting a role in regulating the interaction between AS and caveolin.

**Table 7: Hypothesized Biological Significance of AS Phosphorylation.** Identified AS serine/threonine phosphorylation sites, their hypothesized relevance based on the literature and 3D structure analysis and the number of times that each site was identified in bioinformatic databases.

<table>
<thead>
<tr>
<th>RESIDUE</th>
<th>HYPOTHESIZED BIOLOGICAL ROLE</th>
<th>DATABASE FREQUENCY</th>
</tr>
</thead>
<tbody>
<tr>
<td>T131/S134</td>
<td>Located in the nucleotide domain which is hypothesized to be involved in conformational change upon catalysis. Near cysteine 132, a site known to be nitrosylated under conditions of excess NO production. This site inhibits AS activity. It is possible that the phosphorylation and nitrosylation serve as “on-off” switches in response to cellular NO needs.</td>
<td>3/1</td>
</tr>
<tr>
<td>S180</td>
<td>Located in the catalytic cleft and putatively binds ATP. This site is known to be mutated in citrullinemia, suggesting that it is important for AS activity.</td>
<td>4</td>
</tr>
<tr>
<td>S189</td>
<td>Also located in the catalytic cleft. Forms a hydrogen bond with citrulline so it is important for AS activity.</td>
<td>2</td>
</tr>
<tr>
<td>S328</td>
<td>This site is located in the catalytic domain. It is in close proximity to T131. Additionally, it is adjacent to the AS caveolin binding motif, suggesting a role in regulating this putative interaction.</td>
<td>2</td>
</tr>
</tbody>
</table>
Figure 21 shows the 3-dimensional structure of an AS monomer, dimer and tetramer and illustrates its intricate complexity. The location of the identified phosphorylation sites with respect to the 3-dimensional structure of AS is depicted in Figure 22.

**Figure 21: The AS 3-Dimensional Structure.** AS monomer (A), homo-dimer (B) and homo-tetramer (C) as illustrated by the Structural Genomics Consortium iSEE software (A) or generated by the USF Department of Chemistry (B, C).
**Figure 22: Structure-Function Relationships of AS Phosphorylation Sites.** (A) AS 3D structure (monomer) with location of identified phosphorylation sites in relation to domains (modified from Structural Genomics Consortium iSee software). The N-terminal domain is in green, the catalytic domain is in orange, the C-terminal domain is in gray and the protruding loop that links the N-terminal and catalytic domains is in purple. (B) AS 3D structure (monomer) with location of identified phosphorylation sites in relation to substrates (citrulline and aspartate) (modified from ViewerLite-generated structure). Arrows point to the residues and substrates. Phosphorylated residues are identified in ball and stick form in black. Aspartate and citrulline are in CPK form (gray and purple, respectively).
Mechanism of AS Regulation by Phosphorylation: To understand the role of specific AS phosphorylation sites on enzyme activity, phospho-null (S/T \rightarrow A) and phospho-mimetic (S/T \rightarrow D) variants were generated for T131, S180 and S189, the 3 sites that were confirmed several times by mass spectrometry. First, the effect of alanine mutations was tested. As shown in Figure 23A, preventing phosphorylation at any of these sites significantly reduced the ability of endothelial cells to produce NO. Mimicking constitutive phosphorylation at those sites yielded some recovery of NO production, especially with the S180D variant, but NO levels did not reach those of the wild-type enzyme (Figure 23B). A repeat experiment where the phospho-null and phospho-mimetic variants were compared head-to-head confirmed the observations made when the variants were tested individually (Figure 23C). Since the S180 and S189 have
such essential catalytic functions, a double variant was created where both sites were mutated to alanine. As shown in Figure 23D, the double mutation did not further diminish NO production as compared to the single mutation.

**Figure 23: Role of T131, S180 and S189 on Endothelial Nitric Oxide Production.**

BAEC’s were transiently transfected with wild type (WT) empty vector (EV) or variants (T131A, T131D, S180A, S180D, S189A, S189D, S180A189A). (A) Comparison of WT AS to phospho-null alanine variants \( n = 2 \). (B) Comparison of control (C- untransfected), EV and WT AS to phospho-mimetic (aspartic acid) variants \( n = 1 \). (C) Head to head comparison of EV and WT versus phospho-null and phospho mimetic variants \( n = 2 \). (D) Comparison of control, empty vector and wild type AS to single and double alanine mutants at positions 180 and 189 \( n = 3 \). * \( p < 0.02 \); ** \( p < 0.007 \)
B

Construct

pmol nitrite/mg protein

C

Construct

Fold NO Produced

138
The work with the AS phospho-null and phospho-mimetic variants led to an important observation. Consistently, when BAEC’s were transiently transfected with wild-type AS, there was a high degree of cell loss that increased over time. Theoretically, this was due to the role of AS in maintaining endothelial cell viability by maintaining appropriate amounts of NO as demonstrated in Chapter One. The phospho-null variants of AS never demonstrated any significant cell loss or even visible cell stress. This highly suggested that all 3 sites have a role in AS function.

Another approach that was utilized to assess biological significance of AS serine/threonine phosphorylation involved the use of computational modeling. Since the human crystal structure of AS was solved recently, we now have an invaluable tool to study structure-function relationships. The theory behind this line of experiments was that certain amino acids may enhance or interfere with substrate binding while others might be more accessible to modification by phosphorylation. To test these theories, all the
identified serine or threonine phosphorylation sites [T131 (S131 in human structure), S134, S180, S189 and S328] were modeled plus or minus the phosphate modification and plus or minus substrates. The Molecular Dynamics method was then utilized to measure substrate binding affinity. Serines 180 and 189 were found to flank citrulline. Phosphorylation at either of these sites led to significantly diminished affinity for substrates. In addition, in the tetrameric structure those sites are completely buried in the active site (Figure 24). S131, S134 and S328 lie in close proximity in the 3-dimensional structure (Figure 25). In the dimer, these 3 serines are partially covered by the "free" C-terminal helix that is part of the dimer-dimer interface. Although this region is partially buried, there is surface exposure. S328 has its oxygen facing away from solvent. The oxygen of S131 is hydrogen bonded to H327. S134 has its proton hydrogen-bonded to the backbone carbonyl of H327 leaving its oxygen exposed to solvent and ready for nucleophilic attack. In Figure 26, a close up view of these sites is shown that demonstrates that S134 and S328 are more accessible than S131. S131 has its hydroxyl group buried by the loop where S328 is located. In this figure it is also evident that S131 and S134 are in the outer portion of an alpha helix. In summary, the in silico modeling of AS plus or minus phosphorylation at identified sites revealed that the most likely candidates for modification by phosphorylation are S131, S134 and S328.
Figure 24: Close Up of Human AS Active Site. Serines 180 (left) and 189 (right) are shown in yellow. Aspartate (left) and citrulline (right) are shown in green. ATP would bind in the empty space in the lower left quadrant of the figure.
Figure 25: In Silico Modeling of AS Residues with Good Accessibility for Modification by Phosphorylation. Close up of human AS structure showing S131 (bovine T131), S134 and S328. On top of the figure is the free c-terminal helix that forms the dimer-dimer interface. This helix partially buries these sites.
Figure 26: Close-Up View of S131, S134 and S328. Carbon residues are labeled in gold. S134 and 328 are on the upper left and upper right. S131 is on the lower left.
Discussion

In this Chapter we identified the first biologically significant post-translational modification of AS in the endothelium. The majority of AS regulation has been studied at the level of transcription/translation [10] and very little is known about the post-translational regulation of AS. The work described in this Chapter is the first comprehensive investigation of one post-translational mechanism for regulating AS function.

Although there have not been any post-translational modifications of AS identified in endothelial cells, the post-translational modification of AS was recently noted by Hao et. al [11]. They found that AS is nitrosylated and inactivated in vascular smooth muscle under conditions of excess NO production via iNOS, suggesting that the activity of AS is at least partially responsible for sensing cellular NO levels and adjusting NO output accordingly. This addition of an NO-derived nitrosyl group to the AS protein sequence also suggests a feedback mechanism where NO levels that exceed a certain threshold lead to a decrease in NO output. In addition, in a phospho-proteomics study utilized to identify phosphoproteins in HeLa cells, AS was found to be phosphorylated (S352) [12]. However, the scope of the paper was such that the biological relevance of AS phosphorylation was not explored. In our work, the finding that AS is an endogenous endothelial phosphoprotein was enhanced by the identification of PKA and PKCα as AS kinases and the regulation of this phosphorylation by VEGF.
The importance of tight control of NO production is highlighted by vascular endothelial cells where virtually all phenotypic properties are related to NO bioactivity. Because of this, cardiovascular risk factors often mediate their deleterious effects by compromising these controls which leads to endothelial dysfunction. The finding that PKA and PKCα phosphorylate AS in addition to their known phosphorylation and activation of eNOS [13-18] suggests that during VEGF stimulation, these kinases act coordinately on eNOS and AS via phosphorylation to enhance NO production in endothelial cells. The specificity of the phosphorylation of AS by PKA and PKCα is supported by the fact that Akt, an essential kinase in regulating NO production [19-22], did not phosphorylate AS. Additionally, the substrate-dependence of the reaction and the multiple motifs found in the AS sequence suggest that PKA and PKCα are bona fide AS kinases.

The specific effects of PKA and PKCα on AS function are not clear. Although the evidence at this time compels us to believe that both kinases activate AS, this may in fact not be the case. First, we have to consider the limitations of the tools we utilized to assess whether PKA or PKCα-mediated AS phosphorylation was indeed enhanced by VEGF. Second, we need to be able to test the effect of each kinase on the specific activity of AS. Finally, we need to know specifically which sites are phosphorylated by these kinases. All this work is currently underway.

Although there is still a substantial amount of work that needs to be done to acquire a full understanding of PKA and PKCα-mediated AS phosphorylation, the
literature does support our data and hypotheses. So far, the story with PKA and NO production seems fairly clear. Under many conditions (VEGF, bradykinin, shear stress), PKA increases eNOS phosphorylation at activating sites [13-16]. Our level of understanding of the role of PKCα (and PKC in general) in regulating NO production is not so clear. In many instances, PKC, and PKCα specifically, decrease NO production by a variety of mechanisms [14, 17]. One notable mechanism involves the increase of eNOS phosphorylation during VEGF stimulation by PKC at an inactivating site thus reducing its activity [14]. In fact, in that particular study, PKA and PKC acted reciprocally. On the other hand, a couple of examples link PKCα to enhanced NO production. First, in vivo studies demonstrated that overexpression of PKCα in rat femoral arteries results in an increase in eNOS-mediated blood flow [18]. In another instance, PKCα was shown to be important for the maintenance of vascular integrity during chronic inflammation. This involved the activation of VEGF by decay-accelerating factor [23]. In addition, PKCα enhances eNOS expression [24]. Therefore, it is evident that the phosphorylation of AS by PKCα and PKA is a very significant finding that requires careful and extensive investigation.

There were several other kinases that were not able to phosphorylate AS in vitro in our initial studies, but which warrant continued investigation. AMPK did demonstrate some increase in 32P ATP incorporation, but it was not found to be significant. AMPK has an extremely important role in regulating NO production and energy metabolism [25, 26], two functions it shares with AS. For example, AMPK activates eNOS and thus increases NO production in response to adiponectin, shear stress insulin and VEGF.
stimulation [26-29]. Also, AMPK helps to normalize some of the endothelial defects caused by high glucose by activating eNOS through interactions with HSP90 [30]. AMPK also protects endothelial cells against high-glucose induced apoptosis [31]. Since AS is involved in anti-apoptotic cascades in the endothelium, part of the protection by AMPK could be mediated by AS activation. In addition, the pathways we have linked to AS- insulin, VEGF, TNFα, ceramide and PPARγ- have also been linked to AMPK function [28, 32-35]. We did try two different combinations of AMPK catalytic and regulatory subunits for the in vitro kinase screens and neither gave a significant result. There is the possibility that something present or absent in the in vitro reaction is preventing significant phosphorylation of AS by AMPK. We do hypothesize that if AMPK phosphorylates AS, it is likely an activating modification.

Also, CKII and GSK3β have important roles in regulating NO production but failed to demonstrate the ability to phosphorylate AS in vitro. For example, GSK3β is a downstream target of Akt, a major regulator of eNOS function [36, 37]. In addition, GSK3β regulates angiogenesis in endothelial cells [38, 39]. In one study, it was determined that GSK3β is downstream not just of Akt signaling, but also PKA and MAPK [39]. The mechanism by which GSK3β regulates angiogenesis involves a downregulation of matrix attachment and migration [39]. Considering the regulation of VEGF-mediated NO production by AS activity, it is certainly possible that GSK3β phosphorylates AS. Thus far, eNOS has not been found to be phosphorylated by GSK3β.
In addition, CKII has been shown to phosphorylate calmodulin and this inactivates eNOS [40]. Furthermore, CKII phosphorylates and inactivates protein phosphatase 2A and leads to decreased SP-1 binding to the eNOS promoter and ultimately to decreased eNOS expression [41]. CKII has not been shown to phosphorylate eNOS. An interesting link between CKII and NO metabolism that has not been explored is the fact that it phosphorylates PKA and HSP90 [42, 43], two important proteins that regulate NO production.

Often times, both GSK3β and CKII require their substrates to first be phosphorylated by another kinase before they can phosphorylated their target [44-46]. We believe that these two kinases may indeed phosphorylate AS, but only when priming phosphorylation occurs first. We did attempt to pre-phosphorylate AS with PKCα and then tried the *in vitro* kinase reactions with CKII and GSK3β. We also obtained negative results, suggesting that another kinase needs to pre-phosphorylate AS at sites that are specifically adjacent to the motifs recognized by CKII and GSK3β. Considering the negative regulation of eNOS by both GSK and CKII, it is possible that these kinases may act to diminish AS activity.

Finally, although AS was also not a good substrate for PKG *in vitro*, we did identify this kinase as a possible interacting partner with AS and eNOS in BAEC’s (discussed in Chapter Three). PKG is associated with multiple functions of the endothelium including vasodilation, angiogenesis, improvement of vasodilation during recovery from heart failure and the up-regulation of the mitochondrial oxidative stress...
protection system [47-53]. In addition, PKG co-localizes with eNOS in endothelial caveolae [54], suggesting that it is part of the functional signaling microdomain necessary for citrulline-NO cycle function. Since we have identified AS to be part of this microdomain ([55]; See also Chapter Three), it is quite possible that PKG has direct effects on endothelial AS function. Although most of what is known about PKG regulation of vascular health occurs specifically in smooth muscle, PKG does phosphorylate eNOS at S633 and S1179 [56]. Thus, there is still a possibility that our in vitro kinase screening results are simply false negatives and that there are in fact mechanisms of PKG regulation of AS that occur specifically in the endothelium.

The biological importance of AS phosphorylation by identification of AS kinases is strengthened by our identification of VEGF as one pathway for post-translational regulation of AS. Our finding that AS activity is necessary for maximal activation of NO production by VEGF draws a clear link between AS function and the endothelium-specific biological roles of VEGF such as angiogenesis and vasodilation [28, 52, 57-60]. The decrease seen with the data generated at the two hour time point represented a state where continued stimulation by VEGF allowed for sufficient activation of eNOS and possibly the utilization of compensatory mechanisms so that NO could still be produced at levels significantly above controls. In comparison, when this experiment was conducted at the 10 minute time point, which represented an acute scenario of AS and eNOS activation, MDLA alone led to a dramatic reduction in basal NO production and abolished the VEGF-mediated increase in NO production. This represents a situation where there is insufficient time for additional compensatory mechanisms to emerge in an
effort to restore the cell’s capability to respond to the VEGF stimulation. This also highlights just how critical acute activation of AS is for a suitable response to simulation.

Furthermore, the implication that VEGF stimulates AS phosphorylation is significant due to the known mechanisms by which VEGF leads to activation of eNOS with concomitant increases in NO production [14, 52, 61, 62]. The data showing an increase in AS phosphorylation signal with a phospho-PKA and phospho-PKC substrate antibodies suggests that VEGF leads to the phosphorylation of AS by PKA and PKCα. Although not shown due to difficulty in generating a clear image, it does seem that there are several bands that are near the AS molecular weight that are phosphorylated by PKA and PKC in response to VGEF and other treatments. Additionally, when AS is purified via immunoprecipitation, two bands near the AS molecular weight are noted when a gel is coomassie stained. Therefore, it is possible that there are multiple phosphorylated AS species.

Considering that VEGF and eNOS expression and function are diminished in diabetes and cardiovascular disorders [63, 64], the inclusion of AS in this regulatory scheme suggests that there are multiple unexplored roles of AS in these diseases. This work is supported by the findings of Shen et al. demonstrating that AS activity is essential for endothelial NO production mediated by eNOS (not iNOS) and that inhibiting its activity with MDLA diminishes maximal NO production by the calcium ionophore A23187 [65]. This supports both the functional importance of AS for endothelial NO production and links AS to calcium signaling, which is part of the mechanism by which
VEGF mediates NO production [61]. Additionally, there is reciprocal regulation between NO and VEGF designed to restore homeostasis [66] leading to the distinct possibility that there are mechanisms that will diminish the response of AS to VEGF. Although eNOS is critically important for the regulation of vascular NO production, our work demonstrates that the function of the citrulline-NO cycle as a whole is essential and warrants further study to provide a more global understanding of such an important system.

The regulation of NO production via reversible phosphorylation is exemplified extensively by eNOS. There are at least 5 known sites of eNOS serine/threonine phosphorylation that either activate or inactivate its function. These sites are responsive to a variety of stimuli [67-69]. We have noted a similar mechanism for AS. Our molecular biology studies demonstrated time and time again, that AS is phosphorylated basally and that this changes, qualitatively or quantitatively, upon stimulation. Our mass spectrometry analysis identified 5 sites of serine/threonine phosphorylation and 2 of tyrosine phosphorylation. The phosphorylation at these sites was dynamic and responsive to stimuli. One example was the enhancement of AS phosphorylation at T131 in response to okadaic acid, a serine/threonine phosphatase inhibitor. Specifically, okadaic acid has been shown to diminish the activation of ceramide activated protein phosphatases [70], which suggests a link between ceramide and AS activity in addition to its effects on AS expression demonstrated in Chapter One, Figure 10. If indeed there is a direct relationship between ceramide and the regulation of AS on multiple levels, this would mimic very closely what is known about ceramide and eNOS [71, 72].
There were significant difficulties in obtaining reproducible and reliable data related to quantitation of AS phosphorylation due to the low stoichiometry of phosphorylation, the labile nature of these modifications and possibly due to the methods used to overexpress and purify AS. At times, the precise site that was actually phosphorylated was ambiguous due to close proximity of more than one phosphorylated site. These types of problems are common and this is precisely why these types of projects are known to be extremely difficult to accomplish [73]. We do know, without a doubt, that AS is indeed phosphorylated and we will continue to optimize our methods so that characterization of the function of each relevant phosphorylation site can be achieved. It is possible that one way to solve these issues is to assess organelle-specific AS phosphorylation by, for example, enriching caveolar fractions. There may be distinct areas of the cell where phosphorylation is more prominent and assessing this in whole cell preparations may be diluting the results. This will be further discussed in Chapter Three. Despite the limitations, our collective findings strongly suggest that AS is regulated by a complex interplay of phosphorylation-dephosphorylation events that generate a cellular barcode that defines AS function.

Based on our in silico studies, it is also likely that there are several kinases that phosphorylate AS, mediating the differential signaling necessary to maintain tight control over endothelial NO production. Table 6 lists possible kinases that can phosphorylate AS at indentified phosphorylation sites. Several of them have been discussed in previous sections: PKA (S180), AMPK (T131), GSK3β (S328), and CKII (T131, S180, S189, S328). This section will focus on some of the other kinases identified.
Besides CKII, CKI was also found to be a putative kinase for T131 and S134. There have not been any direct studies of CKI in the nitric oxide system. CKI and CKII have both overlapping and distinct substrate specificities, although they each recognize a different motif in their targets [74]. CKI does phosphorylate glycogen synthase which implies a role in glucose metabolism [75]. Thus, a link to nitric oxide function is possible.

Mitogen activated protein kinase (MAPK) family members were identified as additional putative kinases for T131, S189 and S328. Members of the MAPK/extracellular signal-regulated kinase (ERK) family are known to have multiple roles in regulating NO production. One example is an increase in eNOS expression by angiotensin II which is mediated by MAPK [76]. In addition, MAPK activates PPARγ in response to NO and may represent a cardioprotective mechanism [77]. In addition, MAPK activates eNOS by increasing phosphorylation at S1177 and decreasing phosphorylation at T495 in response to black tea polyphenols [78]. ERK is also involved in mediating the pro-angiogenic effects of NO [79]. Thus, it is possible that at least one MAPK family member is involved in regulating AS activity by phosphorylation. Overall, the combination of in vitro and in silico experiments to identify AS kinases strongly suggested that multiple kinases control the dynamic phosphorylation of AS.

Our investigation of the mechanism of AS phosphorylation by site-directed mutagenesis suggested that T131, S180 and S189 are all important for AS function. This data combined with the in silico modeling of the human AS crystal structure with or
without phosphorylation at all the serine/threonine sites identified by mass spectrometry led to additional implications. First, T131, S134 and S328 are the sites with the highest possibility of being phosphorylated. This certainly supports the mutagenesis data with T131 that showed significantly diminished NO production in the phospho-null variant and partial recovery with the phospho-mimetic variant. It will be interesting to correlate these findings, future molecular biology experiments and the information we have on bioinformatic database frequency. This will allow us to gauge how bioinformatic database frequency relates to true biological significance.

In addition, the decrease in AS-mediated NO production seen with S180 and S189 is most likely due to the direct interactions of these sites with substrates [9]. If phosphorylation can indeed occur at these sites, then the effects we saw are likely due to alterations in substrate binding. Alternatively, even if phosphorylation does not actually occur at those sites, the data we obtained is likely due to the fact that the alanine mutants are missing the hydroxyl side chain of the serine and again this would alter substrate binding and inhibit NO production. Further, although the quaternary structure of AS suggests that these sites are not accessible for phosphorylation, it is possible that they are phosphorylated only in the monomeric configuration. Perhaps phosphorylation of AS monomers is a mechanism that triggers the formation of the active tetramer and this has mechanism has not yet been defined. In addition, even seemingly inaccessible sites may become accessible upon conformational change due to physical interactions with kinases or proteins that can deliver kinases to substrates such as heat shock proteins.
Finally, an intriguing possibility related to AS phosphorylation is the proximity of T131 and S134 to the identified nitrosylation of AS at C132 in vascular smooth muscle [11]. The nitrosylation of this site in endothelium and the putative cross-talk between AS phosphorylation and nitrosylation have not been studied. Furthermore, in endothelial cells, eNOS has been found to be basally nitrosylated (inactive) then de-nitrosylated (active) in response to VEGF stimulation [80]. It is a possibility that a similar mechanism regulates AS due to the important link we made between VEGF signaling and AS function.

There are two other types of post-translational modifications that were not addressed experimentally in this work, but are worth mentioning. First, as discussed further in Chapter Three, acylation is an important mechanism for the subcellular targeting of eNOS with important functional consequences [81]. An in silico search for acylation motifs is the AS sequence suggested that these modifications do not occur in AS. Further, it has recently been determined that eNOS that is phosphorylated at serine 1177 and thus active, is inactivated by glycosylation at this site in diabetes [82]. A global investigation of proteins modified by O-glycosylation found that AS can be glycosylated [83]. Thus, it is clear that the barcode of dynamic post-translational modifications of AS including phosphorylation, nitrosylation and glycosylation illustrate an intricate mechanism of AS regulation that defines its tissue-specific functions and highlights the biological relevance of this enzyme.
In summary, this Chapter has presented the first comprehensive exploration of AS regulation by dynamic serine/threonine phosphorylation. Although deciphering and understanding the phosphoproteome is one of the most complex tasks in proteomics [73], the findings we have to this point have greatly increased our understanding of AS regulation and will open up multiple avenues of important experimentation with far reaching implications for vascular biology.

References


CHAPTER THREE
ARGINOSUCCINATE SYNTHASE SUBCELLULAR LOCALIZATION AND PROTEIN INTERACTIONS

Overview

Argininosuccinate synthase (AS) is an essential mediator of endothelial health by providing a dedicated source of arginine for nitric oxide (NO) production and promoting endothelial cell viability. Our laboratory has previously demonstrated that AS is present in endothelial caveolar fractions along with endothelial nitric oxide synthase (eNOS) and argininosuccinate lyase (AL), the core components of the citrulline-NO cycle. The studies in this Chapter were designed to define the subcellular localization of AS in relationship to these components, to determine whether there are interactions that are essential for nitric oxide metabolism and to begin characterizing additional novel components of the nitric oxide metabolome. Utilizing immunofluorescence microscopy, we found that AS localizes to perinuclear regions and the plasma membrane. Further, eNOS localization overlaps AS localization suggesting functional relevance. We also found that AS colocalizes with caveolin-1, a key regulator of eNOS function, in distinct plasma membrane regions and the Golgi. Utilizing co-immunoprecipitation studies, we found that AS interacts with HSP90 and caveolin-1. These two proteins are intimately
involved in the regulation of endothelial nitric oxide production. We determined that AS and co-localizes with HSP90 utilizing immunofluorescence. We also identified a caveolin binding motif in the AS protein sequence that suggests a direct interaction. Finally, to begin characterizing the nitric oxide metabolome from a more global perspective, we utilized co-immunoprecipitations followed by mass spectrometry. We identified several putative interacting partners that are either novel or understudied in the regulation of NO production. Overall, our work defines a tightly coupled system for the regulation of nitric oxide production and highlights the intricate and dynamic nature of citrulline-NO cycle localization and interactions.

**Materials and Methods**

*Immunofluorescence:* BAEC were plated out on chamber slides. Cells were fixed with 3.7% paraformaldehyde and permeabilized with 0.05% triton X-100. After blocking, cells were incubated with the following antibodies: AS (Everest Biotech), eNOS, HSP90 and caveolin-1 (BD Biosciences). Cells were then incubated with fluorescently labeled secondary antibodies (Invitrogen). Images were generated with a Nikon Eclipse E1000 Fluorescent Microscope running Genus 2.81 software from Applied Imaging. On all experiments, negative controls were conducted by staining one chamber with secondary antibodies only.

*Immunoprecipitation and protein identification using LC-MS/MS:* Co-immunoprecipitation studies were conducted with the following antibodies: AS (Everest
Biotech), HSP90, caveolin-1, eNOS (BD Biosciences), calmodulin I, NOSIP, PKG, AL (Santa Cruz), AMPKα, PKCα, PKAα, CKII, and Akt (Cell Signaling Technology) utilizing the methods described in Chapter Two, Page 101.

For samples that were utilized for mass spec, processing occurred very rapidly due to the labile nature of post-translational modifications and some protein-protein interactions. In addition, RIPA buffer was utilized as the lysis and wash buffer since it is more stringent and diminishes the possibility of non-specific interactions. Eluted protein complexes were subject to SDS-PAGE, bands of interest were excised, then digested proteins were subjected to liquid chromatography-tandem mass spectrometry as described in Chapter Two, Page 108.

**Bioinformatics:** The AS caveolin binding motif was identified utilizing Expasy Prosite (Scan Prosite Tool: http://www.expasy.ch/tools/scanprosite/) by searching for the following motif in the AS protein sequence: [WFY]-X-X-X-X-[WFY]-X-X-[WFY]. The motif was then identified on the 3D crystal structure of AS utilizing ViewerLite software version 5.0 (Accelrys Corporation, San Diego, California).

**Results**

**AS Subcellular Localization Overlaps with eNOS and Caveolin-1:** The subcellular localization of endothelial AS has not previously been characterized and we believe that it is a key regulatory feature of the citrulline-NO cycle. We hypothesized that for optimal
function of the citrulline-NO cycle, AS and eNOS should localize to the same regions within the cell. Utilizing immunofluorescence microscopy, we demonstrated that AS and eNOS colocalize and distribute in endothelial cells in a similar fashion, predominantly in the Golgi and the cytoplasmic membrane (Figure 27). We have previously demonstrated that caveolin-1 and AS co-fractionate in crude caveolar cell extracts, but we did not determine whether these two proteins were in the same or independent caveolar regions. Since eNOS and caveolin-1 colocalize and physically interact, we hypothesized that there would be distinct regions of overlap in the localization of AS and caveolin-1. As shown in Figure 25, AS co-localizes with caveolin-1 in distinct membrane regions and the Golgi, much like what is seen with eNOS.
Figure 27: AS Colocalizes with eNOS and Caveolin-1. Immunofluorescence microscopy images demonstrating the localization of AS in the Golgi and plasma membrane along with its colocalization with eNOS (A) and caveolin-1 (B). AS is red, eNOS or caveolin are green and the merged image shows regions of co-localization in yellow. The nucleus is stained in blue. Arrows point to selected areas of colocalization in the Golgi and plasma membrane (n = 3). (C) Secondary only negative control.
AS Protein Interactions: In order to begin deciphering key AS interacting partners, a series of immunoprecipitations (IP) were carried out. When utilizing AS as the IP antibody, two interactions were consistently noted: HSP90 and caveolin-1 (Figure 28). These interactions were verified by immunoprecipitating with HSP90 and caveolin-1 then probing with AS (Figure 28).

Figure 28: AS Co-Immunoprecipitates with HSP90 and Caveolin-1. (A) Representative blot (n = 3) of BAEC lysates that were immunoprecipitated with an AS antibody (IP AS). The membrane was probed with HSP90 (IB HSP90) and caveolin-1 (IB Cav-1). (B) Representative blot (n = 2) of BAEC lysates that were immunoprecipitated with an HSP90 antibody (IP HSP90). The membrane was probed with AS (IB AS). (C) Representative blot (n = 2) of BAEC lysates that were immunoprecipitated with a caveolin-1 antibody (IP Cav-1). The membrane was probed with AS (IB AS).
These results prompted us to assess the subcellular localization of AS and HSP90. As shown in Figure 29, there appears to be co-localization between AS and HSP90, although the specific regions are difficult to discern since HSP90 is widely distributed in the cytoplasm.

Figure 29: AS Colocalizes with HSP90. (A) Immunofluorescence microscopy images demonstrating colocalization of AS with HSP90 (n = 1). AS is red, HSP90 is green and the merged image shows regions of co-localization in yellow. The nucleus is stained in blue. (B) Secondary only negative control.
Since eNOS has a caveolin binding motif, we decided to search for the same motif in the AS protein sequence. We identified the sequence 317-FAELVYTGF-325 which fits the pattern of the caveolin binding motif found in eNOS: [WFY]-X-X-X-X-[WFY]-X-X-[WFY] (Figure 30). This implies a direct interaction between AS and caveolin.

**Figure 30: AS has a Caveolin Binding Motif.** Three dimensional structure of Human AS (PDB ID 2NZ2) demonstrating the region containing the following caveolin binding motif: [WFY]-X-X-X-[WFY]-X-X-[WFY]. The motif corresponds to the sequence: 317-FAELVYTGF-325. Identified phosphorylation sites and substrates are also shown as described in Figure 22, Chapter Two, Page 135.
It is also important to note that the investigation of AS protein interactions via immunoprecipitation was quite comprehensive and many other proteins were explored. We did obtain some positive preliminary results with AS interactions with eNOS, AL, PKA, PKCα and calmodulin. Taken together, our results demonstrate that there are key protein interactions that potentially mediate AS function and the function of the citrulline-NO cycle as a whole.

Proteomic Examination of the Nitric Oxide Metabolome: In order to begin characterizing the NO metabolome, we utilized immunoprecipitation with an AS or eNOS antibody followed by LC-MS/MS. BAEC cell lysates were collected in RIPA buffer and immunoprecipitation was conducted. Putative interacting components were separated by SDS-PAGE then specific bands were cut from the gel and subjected to mass spectrometry for protein identification. This revealed several interesting interacting partners that are relevant to known regulatory mechanisms of NO production. A list of the most relevant interactions and their known or potential roles in NO biology is shown in Table 8. Some interactions were specific for AS while others were identified with both AS and eNOS antibodies. Interestingly, AS and eNOS did not seem to interact with each other with this methodology, although the interaction has been noted previously with other methodologies (Brenda Flam, unpublished results). Another interaction that was missing from the list was the well documented eNOS-HSP90 interaction. The AS-HSP90 interaction was confirmed with the mass spec data. Table 9 shows all 85 proteins found to co-IP with either AS or eNOS.
Table 8: The Basal Nitric Oxide Metabolome. Table showing a few of the interactions identified utilizing basal cell lysates that were immunoprecipitated with an AS antibody. After separation via SDS-PAGE, bands were excised, digested and subjected to liquid chromatography-tandem mass spectrometry. Table also shows role of these interacting partners in regulating NO metabolism (n = 1).

<table>
<thead>
<tr>
<th>PROTEIN</th>
<th>FUNCTION</th>
<th>IP ANTIBODY</th>
</tr>
</thead>
<tbody>
<tr>
<td>cGMP-dependent protein kinase</td>
<td>Kinase activated by soluble guanylyl-cyclase. Important for vasodilation and regulates eNOS.</td>
<td>AS eNOS</td>
</tr>
<tr>
<td>Dynamin</td>
<td>Large GTP-binding protein residing within similar membrane compartments as eNOS. Interacts with eNOS and increases its activity.</td>
<td>AS</td>
</tr>
<tr>
<td>Golgi SNAP Receptor Complex</td>
<td>Involved in transport from the ER to the Golgi apparatus as well as in intra-Golgi transport. Disruption of intracellular trafficking has been associated with cardiovascular disorders.</td>
<td>AS eNOS</td>
</tr>
<tr>
<td>HSP90</td>
<td>Interacts with eNOS and increases its activity.</td>
<td>AS</td>
</tr>
<tr>
<td>Kininogen-1 precursor</td>
<td>Cleaved into several products, including bradykinin, which is an important regulator of vasodilation. eNOS is known to interact with the bradykinin receptor. This interaction is inhibitory and is released upon treatment with bradykinin.</td>
<td>AS eNOS</td>
</tr>
<tr>
<td>Prohibitin</td>
<td>Mitochondrial protein that protects endothelial cells from reactive-oxygen species damage.</td>
<td>AS eNOS</td>
</tr>
</tbody>
</table>
Table 9: Putative AS and eNOS Interacting Partners. Complete list of all proteins identified via IP with an AS and/or eNOS antibody followed by mass spec as described in Table 8 above.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Species</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin, cytoplasmic 2</td>
<td>Bos taurus (Bovine)</td>
<td></td>
</tr>
<tr>
<td>Trypsin precursor</td>
<td>Sus scrofa (Pig)</td>
<td></td>
</tr>
<tr>
<td>Tropomyosin alpha-1 chain</td>
<td>Bos taurus (Bovine)</td>
<td></td>
</tr>
<tr>
<td>Myosin-10</td>
<td>Bos taurus (Bovine)</td>
<td></td>
</tr>
<tr>
<td>Myosin regulatory light chain 2, smooth muscle isoform</td>
<td>Bos taurus (Bovine)</td>
<td></td>
</tr>
<tr>
<td>Myosin-9</td>
<td>Canis familiaris (Dog)</td>
<td></td>
</tr>
<tr>
<td>Actin-like protein 3</td>
<td>Bos taurus (Bovine)</td>
<td></td>
</tr>
<tr>
<td>Histone H2A type 1</td>
<td>Bos taurus (Bovine)</td>
<td></td>
</tr>
<tr>
<td>Ornithine decarboxylase antizyme</td>
<td>Bos taurus (Bovine)</td>
<td></td>
</tr>
<tr>
<td>Cationic trypsin precursor</td>
<td>Bos taurus (Bovine)</td>
<td></td>
</tr>
<tr>
<td>Actin, alpha skeletal muscle</td>
<td>Bos taurus (Bovine)</td>
<td></td>
</tr>
<tr>
<td>Myosin light polypeptide 6</td>
<td>Bos taurus (Bovine)</td>
<td></td>
</tr>
<tr>
<td>ADP/ATP translocase 2</td>
<td>Tachyglossus aculeatus aculeatus (Australian echidna)</td>
<td></td>
</tr>
<tr>
<td>Vimentin</td>
<td>Bos taurus (Bovine)</td>
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</tr>
<tr>
<td>Kininogen-1 precursor</td>
<td>Bos taurus (Bovine)</td>
<td></td>
</tr>
<tr>
<td>Ribonuclease pancreatic</td>
<td>Hippopotamus amphibius (Hippopotamus)</td>
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<td>Histone H2A type 2-C</td>
<td>Bos taurus (Bovine)</td>
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</tr>
<tr>
<td>Ribonuclease pancreatic</td>
<td>Antilocapra americana (Pronghorn)</td>
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<tr>
<td>Dynamin-1-like protein</td>
<td>Bos taurus (Bovine)</td>
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<tr>
<td>Ubiquitin</td>
<td>Bos taurus (Bovine)</td>
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<tr>
<td>130 kDa phosphatidylinositol 4,5-biphosphate-dependent ARF1 GTPase-activating protein</td>
<td>Bos taurus (Bovine)</td>
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<td>Actin-related protein 2/3 complex subunit 4</td>
<td>Bos taurus (Bovine)</td>
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</tr>
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<td>Myosin-Id</td>
<td>Bos taurus (Bovine)</td>
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<td>Heterogeneous nuclear ribonucleoprotein A1</td>
<td>Bos taurus (Bovine)</td>
<td></td>
</tr>
<tr>
<td>Actin-related protein 2/3 complex subunit 3</td>
<td>Bos taurus (Bovine)</td>
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<tr>
<td>Serum albumin precursor</td>
<td>Bos taurus (Bovine)</td>
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<td>Histone H2B type 1-K</td>
<td>Bos taurus (Bovine)</td>
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<td>Golgi SNAP receptor complex member 1</td>
<td>Bos taurus (Bovine)</td>
<td></td>
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<td>Alpha-S1-casein precursor</td>
<td>Bos taurus (Bovine)</td>
<td></td>
</tr>
<tr>
<td>Glycoprotein GII precursor</td>
<td>Bovine herpesvirus 1.1 (strain Cooper) (BoHV-1) (Infectious bovine rhinotracheitis virus)</td>
<td></td>
</tr>
<tr>
<td>Prohibitin-2</td>
<td>Bos taurus (Bovine)</td>
<td></td>
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<tr>
<td>Abnormal spindle-like microcephaly-associated protein homolog</td>
<td>Ovis aries (Sheep)</td>
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</tr>
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<td>Alpha-enolase</td>
<td>Bos taurus (Bovine)</td>
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<td>Actin-related protein 2/3 complex subunit 1B</td>
<td>Bos taurus (Bovine)</td>
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<td>60S ribosomal protein L4</td>
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<td>Junction plakoglobin</td>
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<tr>
<td>Polymeric-immunoglobulin receptor precursor</td>
<td>Bos taurus (Bovine)</td>
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<tr>
<td>Tubulin alpha-1A chain</td>
<td>Sus scrofa (Pig)</td>
<td></td>
</tr>
<tr>
<td>NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 12</td>
<td>Bos taurus (Bovine)</td>
<td></td>
</tr>
<tr>
<td>Pyruvate kinase isozyme M1</td>
<td>Felis silvestris catus (Cat)</td>
<td></td>
</tr>
<tr>
<td>von Willebrand factor precursor</td>
<td>Canis familiaris (Dog)</td>
<td></td>
</tr>
<tr>
<td>Tyrosine-protein kinase SYK</td>
<td>Sus scrofa (Pig)</td>
<td></td>
</tr>
</tbody>
</table>
An additional piece of data related to protein interactions was uncovered when searching for AS phosphorylation sites via mass spec. When AS was overexpressed and then treated with okadaic acid, T131 was identified as a phosphorylation site (Chapter Two, Figure 19, Page 129). In addition, several other bands were cut out from the gel that contained purified AS and any proteins that might have come down with it. One notable protein identified was NOSIP (eNOS interacting protein). NOSIP is a known binding partner with eNOS and is important for its subcellular localization. NOSIP promotes
translocation of eNOS from the plasma membrane to intracellular sites, thereby uncoupling eNOS from caveolae and inhibiting NO synthesis.

Discussion

In this Chapter, the subcellular localization of endothelial AS was defined, its co-localization with eNOS, caveolin-1 and HSP90 was described and important AS interacting partners were identified. This is the second post-translational regulatory mechanism addressed in this dissertation.

The subcellular localization of AS has been defined in several tissues. For instance, in untransfected or AS-transfected VSMC, AS is detected in both cytosolic and membrane fractions via western blot [1]. AS expression is increased in membrane fractions upon stimulation with LPC/IFNγ, suggesting that AS is transported to membrane regions for functionally relevant purposes [1, 2]. In addition, immunohistochemical studies of AS-transfected cells indicates a punctuate pattern of expression that suggests mitochondrial localization [1], much like what is seen in liver [2]. This study supported the concept of substrate channeling [1], which would necessitate co-localization and assembly into a functional complex. Our identification of AS localization to the perinuclear/Golgi region and plasma membrane supports those previous findings since NO is produced in these two subcellular compartments [3-5]. Thus, much like urea cycle enzymes, the citrulline-NO cycle enzymes form functional
complexes in specific cellular compartments that allow efficient channeling of substrates so that the arginine required for NO production is distinct from bulk cellular arginine.

The distinct localization pattern for AS in endothelial cells also led to an important observation. It is possible that AS has localization-specific or organelle-specific functions. These functions might be related to post-translational modifications. Therefore, some of the difficulties in obtaining definitive mass spectrometry data may be related to the fact that we utilized whole cell lysates to look for AS phosphorylation sites. This may in fact have led to a dilution of the data since perhaps certain regions of the cells have a high population of phosphorylated AS while others, perhaps most, don’t. It will be important to determine the phosphorylation pattern in different cellular compartments to gain a better understanding of the impact and regulation of these modifications.

eNOS is also known to localize to the Golgi and plasma membrane [5] and we did find that AS and eNOS co-localize. Although it is unclear how AS functions within specific cellular compartments, there are two distinct pools of active eNOS, one in the Golgi and one in the plasma membrane. The relative importance of these two pools of eNOS is still under investigation, but one study demonstrated that although NO is produced each of these regions, the plasma membrane produces significantly more NO [3]. In that study, they generated endothelial cells that expressed wild type eNOS, Golgi-only eNOS or plasma membrane-only eNOS without altering any other components of the citrulline-NO cycle. This suggested that either the other enzymes of the cycle are also
present in Golgi and plasma membrane or that there is another source of substrate for eNOS. Our studies and those of others suggest that endothelial cells are dependent on recycling of citrulline to arginine as a source of eNOS substrate [6, 7]. The fact that AS localized to those regions supports those studies. It would be interesting to design a similar study where both AS and eNOS localization could be manipulated. Then, the dependence of co-localization could be definitively proven. However, the studies with Golgi versus plasma membrane eNOS were done by first knocking out endogenous eNOS and then re-constituting the endothelial cells with the different eNOS constructs [3]. We have shown that moderate AS knockdown leads to significant endothelial apoptosis [8], so the same approach might not be successful for AS. This suggests that while the function of eNOS may be exclusively to produce NO, AS might have several different functions, including as anti-apoptotic signaling [8], that make it even more important for overall vascular biology.

In addition, we demonstrated that AS localized in distinct plasma membrane regions in conjunction with caveolin-1 [7, 9]. This expands our previous work by demonstrating that the regions where AS localizes in the plasma membrane are also regions where caveolin-1 is present. In other words, our previous work showing that AS and caveolin localized to similar crude caveolar fractions did not prove that the localization was to the same specific caveolar regions [9]. The co-localization of AS and caveolin suggests a role for caveolin-1 in assembling AS into a functional signaling microdomain, much like its role in regulating eNOS transport and function. For example, caveolar domains assemble at the Golgi and traffic to the plasma membrane as stable
transport platforms [10, 11]. These platforms constitute signaling microdomains where multiple kinases, phosphatases and regulatory enzymes assemble into functional complexes [12, 13]. The transport mechanisms regulating the formation of the NO metabolome at caveolae enhance NO production at the plasma membrane and involve vesicular transport elements and cytoskeletal components [10, 11, 14-17].

In addition, interaction with other proteins plays a role in eNOS targeting. For example, there are two eNOS-interacting proteins named NOSIP (for eNOS-interacting protein) [18] and NOSTRIN (for eNOS-trafficking inducer) [19], which both influence the subcellular localization of eNOS. NOSIP, targets eNOS to the cytoskeleton and inactivates it [20]. NOSTRIN, when overexpressed, leads to translocation of eNOS from the plasma membrane to intracellular vesicular structures [19], possibly involving an endocytic process [21]. Utilizing AS overexpression, purification and mass-spectrometry of associated proteins we did identify NOSIP as a putative binding partner of AS. This leads to the intriguing possibility that there are some shared mechanisms in AS and eNOS transport. Although these findings could not be confirmed utilizing IP/western, the antibodies for NOSIP that we utilized were not very good, our IP techniques needed optimization and the AS antibody available on the market at that time did not work well for IP. With the new methods and tools we now have available, it is certainly worthwhile to pursue this mechanism.

Another mechanism that regulates the subcellular translocation of eNOS is dual acylation, an irreversible N-myristoylation at Gly2 and reversible thiopalmitoylation at
C15 and C26 [16]. These lipid functional groups are responsible for targeting eNOS to the Golgi and plasma membrane, particularly to caveolae [16, 22, 23]. Acylation deficient eNOS variants cannot translocate to the plasma membrane and this leads to alterations in eNOS activity [14]. It was intriguing to hypothesize that AS might be modified by acylation. A bioinformatic search for motifs for myristoylation and palmitoylation did not identify any such motifs in the AS sequence. It is still possible that AS is acylated via motifs that have yet to be described. In fact, the palmitoylation motif uncovered for eNOS is distinct from the motif found on other signaling proteins [22]. For example, the motif identified for the Src family of proteins is MGCXXC/S while eNOS the motif for eNOS is MGXXXS…C15(GL)3C26. The human AS sequence does have a glycine at position 5 and a cysteine at position 19, making it possible that lipidation does indeed occur. Although we did not examine the transport of AS in this work, the findings we describe below do support a dynamic translocation of AS in endothelial involving several key interacting partners.

We believe that our evidence supports a model whereby protein interactions regulate the subcellular localization of AS. Caveolin-1 may indeed be a key factor in this level of regulation. The fact that the AS protein sequence has a caveolin binding motif suggests that the interaction we found via immunoprecipitation may in fact be a direct interaction. In addition, previous unpublished data from our laboratory supports this hypothesis since both GST and His-tag pull down experiments demonstrated that AS can pull down caveolin-1 (Brenda Flam, unpublished results). Since this motif has been identified, it can be utilized for the design of experiments aimed directly at disrupting the
motif and determining not only whether the interaction is direct, but also the specific region that is involved in the interaction. Since we have identified an AS phosphorylation site very close to that motif, it is quite possible that phosphorylation regulates this interaction. The eNOS caveolin binding motif is located in the peptide 348-FPAAPFSGW-356 [24, 25]. To date, there have not been any eNOS phosphorylation sites in the vicinity of the caveolin binding motif with respect to the secondary structure. Since the entire eNOS protein has not been crystallized, it is difficult to know if perhaps one of the eNOS phosphorylation sites is near the caveolin binding motif in the 3-dimensional structure. However, it is known that phosphorylation regulates the interaction between eNOS and caveolin-1, thus it is certainly possible that the mechanism involves spatial proximity of phosphorylation sites. We speculate that there is an interrelated mechanism that controls the binding of caveolin to AS and eNOS.

Along with the possible regulation of AS localization and function by caveolin-1, the finding the HSP90 also co-localizes with and interacts with AS strengthens our hypothesis due to the reciprocal regulation of NO production by these two proteins. HSP90 is involved in the activation of eNOS by allowing the interaction of eNOS with calmodulin, the phosphorylation of eNOS by Akt and the dissociation of eNOS from caveolin-1 [26-29]. It is possible that the AS-HSP90 interaction regulates AS through similar mechanisms. Interestingly, we were able to confirm this interaction with IP/mass spec analyses.
Several more interactions were uncovered via IP analyses, including the interactions of AS with AL, eNOS, calmodulin, PKA and PKCα, but the results have not been reproduced. Previous work in our lab did indicate a direct interaction between AS, AL and eNOS (Brenda Flam, unpublished results) and our preliminary results do support this. In addition, the interaction of AS with PKA and PKCα supports previous studies that demonstrate the presence of both of these kinases in caveolae [30, 31]. Considering that our results show that AS is an *in vitro* substrate for these kinases, these interactions are certainly worth pursuing. The possible interaction of AS with calmodulin suggests regulation via calcium signaling, and our link between AS and VEGF supports this since VEGF stimulates NO production, in part, by increasing intracellular calcium [26]. Overall, the above findings strengthen our hypothesis of a dynamic and complex set of interacting proteins in the NO metabolome.

Up to this point, our results were obtained by rational investigation of AS interacting partners based on preliminary data or the known role of certain proteins in regulating NO production. Our ultimate goal, was to put together the pieces of the nitric oxide metabolome. In order to accomplish this, we needed a more global approach to characterize the dynamic nature of both hypothesized interactions and novel or understudied interactions. We developed a proteomic approach to identify all proteins that are pulled down when an AS or an eNOS antibody was used for IP analyses. We identified 85 proteins that co-immunoprecipitated with AS and/or eNOS. From those, there were 6 that stood out as having a possible functional role.
First, cGMP-dependent protein kinase or protein kinase G (PKG) was found to interact with both AS and eNOS. PKG is an important kinase in regulating the function of nitric oxide in vascular smooth muscle. When endothelial NO is released, it acts in smooth muscle to activate soluble guanylyl cyclase (sGC), increase cGMP and activate PKG. This leads to vasodilation [32]. PKG has been also been found to have direct functions in the endothelium. For example, PKG colocalizes to caveolae in both smooth muscle and endothelium [30]. In addition, PKG is involved in the regulation of angiogenesis in endothelial cells [33]. Considering the link between VEGF and AS that we established in Chapters One and Two and the well established link between VEGF and eNOS [26, 33-41], it is quite possible that this is a true functional interaction. This interaction was not verified by IP/western, but optimization of the IP methodology or a different methodology altogether might reveal a different result.

A second interaction revealed by IP/mass spectrometry was dynamin. Dynamin is a large GTP-binding protein that co-localizes with and interacts with eNOS, thereby increasing its activity [42]. It targets to Golgi membranes and also co-localizes with caveolin in caveolae [43, 44]. In addition, NOSTRIN (eNOS trafficking inducer) interacts with dynamin and mediates eNOS subcellular translocation [21]. In our experiments, dynamin was not found to interact with eNOS. It is possible that the conditions and/or methods used did not favor this interaction. The interaction between AS and dynamin fits well with previous, unpublished data from our lab where tandem affinity purification was utilized to pull down AS and associated proteins. These results identified a GTP-binding protein as an AS interacting partner.
Another AS and eNOS interacting partner identified via IP/mass spectrometry was the Golgi SNAP receptor complex. This protein is one of several involved in transport from the endoplasmic reticulum to the Golgi and in intra-Golgi transport [45]. It has been demonstrated that in vascular disorders, there are disruptions of intracellular trafficking. Specifically, hypoxia and other conditions cause disruptions in ER/Golgi trafficking that lead to sequestration of eNOS and a reduction in plasma membrane associated eNOS. This leads to diminished NO production [46]. The possibility of AS interacting with this protein suggests that AS trafficking in the ER and Golgi may be an important regulatory mechanism.

We also identified HSP90 as an AS interacting partner via IP/mass spec, which confirms the IP/western data described earlier. The fact that we did not find the well documented eNOS-HSP90 interaction highlights the fact that each methodology utilized to probe for protein interactions, and the variations within individual methodologies, can lead to false positive or false negative results.

Kininogen-1 precursor was another AS and eNOS interacting partner identified via IP/mass spectrometry. This precursor is cleaved into several products including bradykinin [47]. Bradykinin is a vasodilator that regulates eNOS function via several mechanisms [48-51] and the coordinate interaction with both AS and eNOS suggests that bradykinin also plays an important role in regulating AS function.
The final protein in the IP/mass spectrometry analyses that has an important functional link in the endothelium was prohibitin-1. It interacted with both AS and eNOS. This protein was originally identified in yeast and is localized to the inner mitochondrial membrane. It is now apparent that prohibitins have diverse roles in several disease states such as obesity and inflammation [52]. Recently, it was determined that prohibitin-1 is highly expressed in the vascular system [53]. Knock-down of prohibitin-1 in endothelial cells leads to dysfunction characterized by increased production of reactive oxygen species in mitochondria. This suggests that prohibitin-1 is important for protecting endothelial cells from oxidative damage [53]. Interestingly, prohibitin was recently found to regulate the function of OPA-1, a dynamin-like protein involved in cristae morphogenesis [54]. This regulation led to the control of cell proliferation and apoptosis and the authors speculated a role for prohibitin in lipid rafts. Since AS has been hypothesized to localize in mitochondria in vascular smooth muscle [1] and does localize to mitochondria in other tissues [2], it is possible that AS is also localizes in endothelial cell mitochondria. There is some evidence that eNOS localizes to mitochondria [55] but the function of the citrulline-NO cycle in endothelial mitochondria has not been studied. Furthermore, NO itself has important roles in regulating mitochondrial function and biogenesis [56, 57]. Collectively, the identification of dynamin and prohibitin and AS interacting partners implies important functional significance. Thus, a functional citrulline-NO cycle in mitochondria might regulate functions that are specific to that organelle.
Finally, another set of prominent interactions seen via IP, IP/mass spec and tandem affinity purification/mass spec were several cytoskeletal components including actin, vimentin and tubulin. Although the high expression of these proteins in most cell types leads to the initial assessment that these results are false positives, it is quite possible that there is an intricate cytoskeletal network responsible for the trafficking of AS within endothelial cells. Indeed, such mechanisms have been extensively characterized for eNOS. There is a prominent role of actin polymerization that regulates eNOS activity and transport [58]. More recently, it was uncovered that the protein α-actinin-4, an actin binding protein responsible for actin cross-linking, interacts with and inactivates eNOS by competitively inhibiting calcium-dependent activation [59]. Furthermore, the mechanisms of eNOS regulation by NOSIP and NOSTRIN involve the actin cytoskeleton [18, 19]. Shear stress regulates dilation and remodeling of resistance arteries via several cytoskeletal components such as vimentin, desmin and intermediate filaments [60]. Thus, further investigation into the role of the actin cytoskeleton and other cytoskeletal components in AS-eNOS co-translocation and function is an important area for further exploration.

It is clear from our studies that obtaining an accurate picture of true interacting partners is a complex process. Each technique employed can lead to false positive or false negative results. In addition, many techniques cannot confirm whether an interaction is direct or indirect. Despite these limitations, our studies indicate that there is a vast and dynamic network of associating proteins that regulate the function of the nitric oxide metabolome.
References


PERSPECTIVES

Summary

The work conducted in this dissertation has uncovered significant advances in our understanding of AS regulation as it relates to nitric oxide production and vascular health. In Chapter One, the regulation of AS function and expression was explored. The data revealed the important role of AS in the production of nitric oxide since overexpressing AS led to a significant increase in endothelial NO production above the levels of the endogenous enzymes of the citrulline-NO cycle. We were also able to show that insulin and VEGF up-regulated AS expression and increased NO production while ceramide diminished AS expression. In Chapter Two, we identified and characterized the first post-translational modifications of AS in the endothelium: phosphorylation at serine, threonine and tyrosine residues. After utilizing bioinformatics to determine that AS phosphorylation was a possible AS regulation mechanism, we focused on biological relevance. First, we identified PKA and PKCα as kinases that regulate AS phosphorylation. We also showed that phosphorylation of AS by PKA and PKCα is required for maximal VEGF-mediated NO production. We then identified 7 different sites of phosphorylation utilizing a proteomics approach and demonstrated the potential biological roles of T131/S134, S180, S189 and S328. In Chapter Three, the subcellular
localization of AS was defined in endothelial cells for the first time. In addition, our results demonstrated an overlap in localization of AS with eNOS, caveolin-1 and HSP90. We were then able to characterize important AS interacting partners utilizing immunoprecipitation including caveolin-1 and HSP90. We also provided the first example of the possible relationship between AS phosphorylation and protein interactions utilizing the proximity of S328 to the newly identified caveolin-binding motif. Finally, utilizing proteomics, we were also able to identify 85 proteins that interact with eNOS and/or AS in BAEC’s under basal conditions. From that list of proteins, 6 were highlighted as having known or potential roles in regulating NO production: PKG, dynamin, prohibitin, HSP90, the Golgi SNAP receptor complex and kininogen-1.

**Significance**

The work presented in this dissertation demonstrated that AS is a central player in the regulation of NO production as evidenced by the multiple and intricate mechanisms that regulate its function. Often times, the studies that focus strictly on eNOS function to address the regulation of NO production only tell part of the story. Considering that most phenotypic properties of the endothelium are mediated by NO [1], it is essential that the scientific community focus more attention on looking at the bigger picture.

The key observation that in the endothelium, AS transient overexpression enhances nitric oxide production further supports the fact that AS is the rate limiting step in the process [2-5]. Such an important distinction supports our initial hypothesis that
multiple-levels of AS regulation need to be in place in order to maintain NO levels at the appropriate levels. The finding that AS overexpression led to a loss of endothelial cell viability confirms the importance of AS as an anti-apoptotic signaling molecule [6] and implies that the specific mechanisms by which AS regulates this process warrant further investigation.

The transcriptional regulation of AS is important in many of its key target tissues such as liver, immune cells and macrophages [7-11]. Our work demonstrating the coordinate regulation of AS and eNOS expression by both positive and negative stimuli extends our understanding of the importance of this mechanism in regulating the health of the endothelium. This also opens avenues for investigation of these observations in animal models of vascular disease. In addition, this work continues to add to the mounting evidence that the arginine paradox can be explained by the tightly coupled arginine regeneration system exemplified by the citrulline-NO cycle [12-14].

The link between AS and VEGF is the first demonstration of a potential role of AS in mediating angiogenesis. The possible direct effects of AS on the angiogenic process could have dramatic importance in understanding the global effects of AS on vascular health. Furthermore, this link may explain some of the possible roles for AS in cancer [15]. Additionally, our work links AS to calcium signaling thus opening up another possible regulatory avenue.
Post-translational regulation of AS, whether by phosphorylation or protein interactions, is an essentially uncharacterized field of NO biology. Considering the fact that NO is such a potent mediator of whole body metabolism [16, 17], it is certainly a rational principle that multiple mechanisms are needed to maintain the function of the system. Post-translational modifications allow for acute regulation of protein function. It is absolutely essential for AS to be able to respond quickly to the ever changing cellular need for NO, and we have shown that AS phosphorylation is one such mechanism. This type of regulation also opens up a multitude of therapeutic avenues aimed at the kinases and phosphatases that control the ever-changing barcode of post-translational modifications. Considering that eNOS is so intricately regulated by dynamic association with a number of other proteins [17], it makes sense that AS is part of this regulatory scheme. In addition, the continuation of our work to characterize the NO metabolome will be essential in identifying additional, novel binding partners that have not previously been considered.

The multiple possible functions of AS in the vasculature and the complex and numerous mechanisms that regulate its function, some presented in this dissertation and many yet to be uncovered, lead to the prospect that many possible interventions to regulate AS function could impact a number of disease processes. For example, there is controversy related to the usefulness of arginine supplementation in the treatment of vascular disorders [18]. Our work suggests that this is because of the multiple functions of AS in vascular and non-vascular roles. Although citrulline supplementation may indeed be a better option due to the unique metabolic fate of this amino acid as compared
to arginine [19], it remains to be determined whether whole body supplementation will be
effective since increasing the substrate for AS might have additional, unwanted effects
such as enhanced NO production in tissues that do not require it or perhaps even
pathogenic angiogenic consequences related to tumorigenesis. Perhaps the delivery of
citrulline to the specific tissues with a deficiency in NO production is a better option.

In addition, our finding that AS is phosphorylated by kinases that are also
important for eNOS regulation improves our understanding of how drugs that target some
of those kinases might have a greater cardiovascular impact than originally intended. In
addition, targeting kinases that directly phosphorylate AS for therapeutic purposes might
have greater efficacy and fewer side effects since the target is further downstream in the
NO signaling cascade. Another therapeutic avenue involves protein interactions. If a
defect in the HSP90 interaction with AS and eNOS can be corrected therapeutically with
a drugs such as insulin sensitizers that have been shown to correct this defect [20], the
benefit to the vascular system could be prominent. The use of such specific therapies will
depend on genomic and proteomic approaches that will allow us to understand the
specific defect that is causing an individual’s disease. Whether the therapy is
pharmacological or nutritional, this type of information will allow for the implementation
of individualized patient therapies that will dramatically improve our success in the
prevention and treatment of the prominent and devastating diseases that plague global
health.
Limitations

One of the main limitations of our work is the fact that it has almost entirely been carried out in tissue culture. There are definite advantages to tissue culture systems since they allow for the study of single variables without the influence of some compensatory mechanisms and of other tissues. These studies also allow for tissue specific mechanisms to be defined. Although tissue culture does not completely mimic an *in vivo* approach, it does allow for initial hypotheses and mechanisms to be developed that can then be used as starting points for translational work. We have begun projects to translate our work into relevant animal models with an ultimate goal of determining the applicability of our work in humans. However, our work and that of others fits well with the known functions of eNOS in the vasculature which has been extensively characterized in animals and humans. Therefore, we feel strongly that our tissue culture work will have relevance for human health.

Another limitation of our work relates to the groundbreaking nature of our projects. For several of the experimental avenues that were undertaken, adequate tools were not readily available. Thus, methodologies had to be designed to make judicious use of available tools. In addition, for the continuation of this work, new tools will have to be generated such as site-specific AS antibodies and ultimately even tissue-specific AS knockout mouse models so that a thorough understanding of the role of AS in human diseases can be delineated. These tools, in conjunction with similar tools already
available for eNOS, will allow for a thorough understanding of the importance of AS within the citrulline-NO cycle.

**Future Directions**

There are several areas of work that will be important in expanding the findings of this dissertation. In Chapter One, each pathway studied requires two main areas of investigation to clearly delineate the role of AS expression in NO production. First, the role of insulin, VEGF and ceramide on AS specific activity needs to be determined. That will allow the direct connection between these biological molecules and AS function. Although this was addressed partially with the AS inhibitor studies with VEGF, enzyme assays are critical to make stronger conclusions. Second, the regulation of AS expression needs to be mechanistically defined by carrying out experiments that will determine whether message stability or specific promoter regulation accounts for the up or downregulation of AS. It is also possible that the mechanisms involving AS regulation by ARP (Argininosuccinate Synthase Regulatory Protein) might be tied into the transcriptional regulation uncovered in Chapter One.

The findings Chapter Two related to AS post-translational regulation by phosphorylation are indeed novel and could drive the field of nitric oxide biology into multiple new and relevant areas of investigation. Ultimately, progress in this field will depend on definitively identifying specific sites of AS phosphorylation that are necessary for NO production and importantly, for AS specific activity. This will require continued
optimization of proteomic approaches, the development of AS enzyme assays and the
generation of site-specific AS antibodies. Currently, experiments are underway to
identify the specific sites phosphorylated by PKCα and PKA and also to assess AS
phosphorylation in the endogenous AS enzyme via immunoprecipitation. In addition, the
possibility of other types of PTM needs to be explored such as nitrosylation,
glycosylation and acylation. It has taken many, many years to get a decent understanding
of these regulatory mechanisms for eNOS function. Even with all the progress, there are
still many areas that require further research and understanding. Certainly, it will take a
comparable amount of time and concerted effort to get a strong understanding of how
similar mechanisms regulate AS.

The regulation of AS by protein interactions is also of great significance. A more
thorough understanding will require both basic molecular and proteomic approaches.
Each individual protein interaction will have to be characterized extensively. First, the
utilization of several types of methods such as pull down approaches will be necessary to
confirm the interactions and to determine if the validated interactions are direct or
indirect. Second, to further prove whether interactions are direct or indirect, studies will
need to be designed to determine the specific regions of each protein involved in the
interaction. We already have a good start with the caveolin-AS interaction due to the
identification of a caveolin-binding motif in the AS protein sequence. The biological
significance of these interactions will need to be assessed by determining the role of each
interaction on AS specific activity. Finally, the optimization and continuation of
proteomic approaches to find additional members of the nitric oxide metabolome will be
essential to truly understand nitric oxide metabolism from a global systems perspective. Furthermore, these approaches will also allow us to define the role of PTM in regulating protein interactions via the utilization of methods that can simultaneously identify protein interactions and PTM. We have already designed these methodologies in conjunction with the Moffitt Proteomics Core and preliminary experiments have been initiated.

Finally, the significance of our work will be strengthened by projects designed to characterize our tissue culture findings in animal models and ultimately in humans. We have such a project underway currently. We plan on beginning our translational work by utilizing streptozotocin (STZ) to induce diabetes in rats. This type 1 diabetes model will allow us to examine the impact of total insulin deficiency on the expression of AS, AL and eNOS. This type of work will mimic our tissue culture experiments with insulin. In addition, we will have 4 treatment groups: sham treated, STZ only, STZ plus suboptimal insulin treatment and STZ plus optimal insulin treatment. This will allow us to study expression patterns in conditions of normal glycemia, moderate hyperglycemia and extreme hyperglycemia. We will also be able to measure serum NO levels, the expression of other proteins that we have identified as essential for AS regulation (VEGF, PKA, HSP90, etc.), and the subcellular localization of these proteins. Therefore, we can build an in vivo model of specific dysfunction of the citrulline-NO cycle in diabetes as it relates to AS that can eventually be utilized to carry out human studies.
References


APPENDIX A: Related Publications

TROGLITAZONE UP-REGULATES VASCULAR ENDOTHELIAL ARGININOSUCCINATE SYNTHASE

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Abstract

Vascular endothelial nitric oxide (NO) production via the citrulline-NO cycle not only involves the regulation of endothelial nitric oxide synthase (eNOS), but also regulation of caveolar-localized endothelial argininosuccinate synthase (AS), which catalyzes the rate-limiting step of the cycle. In the present study, we demonstrated that exposure of endothelial cells to troglitazone coordinately induced AS expression and NO production. Western blot analysis demonstrated an increase in AS protein expression. This increased expression was due to transcriptional upregulation of AS mRNA, as determined by quantitative real time RT-PCR and inhibition by 1-D-ribofuranosylbenzimidazole (DRB), a transcriptional inhibitor. Reporter gene assays and EMSA analyses identified a distal PPARγ response element (PPRE) (–2471 to –2458) that mediated the troglitazone increase in AS expression. Overall, this study defines a novel molecular mechanism through which a thiazolidinedione (TZD) like troglitazone supports endothelial function via the transcriptional up-regulation of AS expression.
Introduction

Almost all normal functions of vascular endothelial cells are dependent on or affected by the bioactivity of nitric oxide (NO). Thus, impairment of endothelial NO production is often a common pathogenic mechanism by which cardiovascular risk factors such as hypercholesterolemia, hypertension, smoking, homocystinemia, vascular inflammation, and diabetes mellitus promote their deleterious effects on the vascular wall [1]. Endothelial NO production is supported by reactions catalyzed by endothelial nitric oxide synthase (eNOS), argininosuccinate synthase (AS) and argininosuccinate lyase (AL) which are core components of the citrulline-NO cycle [2-4]. The principal role of AS and AL catalysis is in the conversion of citrulline to arginine, the substrate utilized by eNOS to produce NO and citrulline. AS is rate-limiting to the citrulline-NO cycle [3, 4], and as such is required to sustain endothelial function and viability [5].

PPARγ is a member of the nuclear receptor superfamily of ligand-activated transcription factors that has been shown to regulate the transcription of genes involved in lipid metabolism, differentiation and cell growth [6]. Both naturally derived PPARγ ligands, including a number of fatty acid metabolites such as eicosanoid derivatives [7] and 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) [8, 9], as well as synthetic ligands such as the thiazolidinediones (TZDs) have been described. The TZDs have insulin-sensitizing properties [10-12] which provide cardiovascular benefits [13-17] and promote flow-mediated vasodilatation, in part, by stimulating endothelial NO production via the activation of eNOS [17, 18]. Because of these findings, we examined whether the TZD,
troglitazone, known to promote NO production [19] and vasodilatation in diabetic patients [20], may affect the efficiency of the citrulline-NO cycle via AS expression in vascular endothelial cells.

Materials and Methods

Cell Culture: Bovine aortic endothelial cells (BAEC) were cultured in complete Dulbecco’s modified Eagle’s medium (1 g/L glucose, Mediatech) containing 10% fetal bovine serum (Hyclone Laboratories), 100 units/ml penicillin and 100 μg/ml streptomycin (Mediatech) at 37°C in an atmosphere of 5% CO₂.

Nitric Oxide Assay: BAEC were treated with troglitazone as indicated in DMEM (minus phenol red) plus 5% fetal bovine serum. Aliquots (100 μl) of media were removed at the indicated times and nitrite was measured as an indicator of cellular NO produced using a fluorometric method [21]. Samples were read on a BMG Fluostar Galaxy spectrofluorometer in a 96-well plate. Data is presented as quantity of nitrite produced in pmols per mg protein.

Western Blot Analysis: Following treatment with troglitazone, BAEC were harvested in 500 μl PBS, centrifuged briefly and lysed in RIPA buffer. The lysate was incubated on ice for 30 minutes and protein concentration determined by BCA reagent (Pierce). Ten μg of protein was electrophoresed on 4-15% polyacrylamide gels (Bio-Rad) and transferred onto membrane (Immobilon-P). Membranes were incubated with
antibody 1:2500 anti-AS and 1:1000 anti-GAPDH (BD Transduction Labs) in 5% blocking solution in TBS-T (20 mM Tris-HCl, 137 mM NaCl, 0.1% Tween-20) and then washed in TBS-T. Membranes were subsequently incubated with horseradish peroxidase-conjugated anti-mouse antibody for 1 hour, immersed in ECL reagent (GE Healthcare) for 1 minute and then exposed to film. Band intensities were quantitated using ImageQuant software (Molecular Dynamics).

**RNA Isolation and Quantitative RT-PCR:** Total RNA was isolated using Tri Reagent following the manufacturer’s instructions (Sigma). RNA was treated with DNase (Ambion DNA-free). Five hundred ng of RNA was reverse transcribed using Superscript II (Invitrogen) as described previously [22]. Real time quantitative PCR was performed using AS specific primers ASL228 and ASR278 [22]. Results were normalized to 18S rRNA.

**Vector Construction:** Luciferase reporter constructs were designed to include the AS promoter and 5′-UTR up to the AUG start codon cloned upstream of the luciferase gene. Luciferase reporter construct p3ASP189 was described previously [23]. Left primers ASL-3075 (5′-GTACCTCCACTGAAATTGAA) and ASL-2616 (5′-GCACCTGAGGAAAGCTCAAAGGCCATGGTG) were combined with ASRluc, (5′-ATAGAATGGCGCCGGGCGTTTCTTTATGTTTTTGGCGTCTTCCATCGTGACGG GTGACCAGCGGC) to amplify a deletion series of the AS promoter with an Xho I site on the 5′ end and an Nco I site on the 3′ end which were used to clone into the vector
pGL3Basic (Promega) and create the vectors p3ASP3075 and p3ASP2616, respectively. Mutations were made in the PPRE sites in p3ASP2616 using a three-way PCR method [23]. Primer PPREmut (5’-GCTGGTCTTGTGTCTCTCTCTCTATCAGGTGA) was combined with primer ASRluc to amplify a fragment that contained the mutations. This PCR product was then used as a right primer and paired with ASL-2616 to produce a second product. A third round of PCR was used with the second product as a template with primers ASL-2616 and ASRluc to enrich for the target. Amplified products were purified and ligated into pGL3Basic to create p3ASP2616PPREmut. All constructs were verified by sequencing.

*Luciferase Assay Analysis*: BAEC were cultured as described above and plated in a 24 well plate prior to transfection. Experimental plasmids (200 ng each) and renilla control plasmid pRL-TK (50 ng) were transiently transfected into BAEC using Transit-LT1 (Mirus) in serum free media. Transfected cells were cultured for 24 hours in media containing troglitazone and lysed in passive lysis buffer (Promega). Ten lysate was assayed for luciferase and renilla activity using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions.

*Electrophoretic Mobility Shift Assay*: Nuclear extracts, prepared from BAEC as described previously [23, 24], were combined with or without cold oligonucleotide competitors and incubated for 20 minutes at room temperature. Probes were labeled by combining equimolar amounts of complementary oligonucleotides (2x10^{-10} moles),
APPENDIX A: (Continued)
which were heated to 70°C, and allowed to cool to room temperature slowly. The oligos
were labeled using 10 μl [α-32P]dCTP (3000 Ci/mmol) and Klenow enzyme.
Unincorporated label was removed using Nuc Away spin columns (Ambion). The
reaction mixture contained binding buffer (10 mM HEPES, pH 7.9, 10% glycerol, 1 mM
DTT, 0.1 μg/μl poly(dI:dC), 0.5 μg/μl BSA and 4000 dpm/μl radiolabeled probe) and
nuclear extract (5 μg) in a total volume of 10 μl which was incubated at 30°C for 30
minutes. Samples were loaded onto a 5% non-denaturing polyacrylamide gel and run at
180 V. Gels were dried under vacuum and exposed to film. Double-stranded
oligonucleotides composed of the following sequences were used for EMSA analysis:
PPRE (5’-ACCTGAGGTCAGGAGTTCAAGACC-3’), PPREmut (5’-
ACCTGAGAACAGGAGAACAAGACC-3’), Sp1 site 1 (5’-
GCTCCAGGCGGGGGCGGGGGCGGGGGCG-3’), Sp1 site 2 (5’-
GGCCGGCGGCGGCGGGTGCTGTGCGGC-3’) and Sp1 site 3 (5’-
CCGGTCACCGGCCTGCCCGGCGCCCTG-3’).

Statistical Analyses: Experimental data is expressed as the mean of experiments
plus or minus the standard error of the mean. Each experiment was performed
independently at least three times.

Results

The PPARγ Ligand, Troglitazone, Increases Endothelial NO Production: To
confirm that troglitazone stimulates NO production in cultured endothelial cells,
confluent bovine aortic endothelial cells (BAECs) were incubated for 24 hours with increasing concentrations of this synthetic PPARγ agonist. As shown in Figure 1A, a dose-dependent increase in NO production following treatment was observed up to 20 µM troglitazone. The dose dependent effect was consistent with previous findings relative to the extent of NO produced [18].

**Troglitazone Treatment Increases AS Expression:** Since the expression of AS is necessary to support endothelial NO production [3-5, 25], we investigated whether troglitazone affected the increase in vascular endothelial NO production, at least in part, through the up-regulation of AS expression, or whether the increase in NO production was simply due to established effects on eNOS activation [17-19]. Confluent BAECs were treated with increasing concentrations of troglitazone for 24 hours and AS protein levels were determined by western blotting. As shown in Figure 1B & 1C, treatment with troglitazone resulted in an increase in AS protein that closely correlated with the troglitazone dependent increase in NO production, demonstrating that this PPARγ agonist does indeed support an increase in NO production through up-regulation of AS expression.

To determine whether the increase in AS expression resulted from transcriptional upregulation, BAECs were grown to confluence and stimulated with troglitazone for 24 hours. RNA was prepared and quantitative real time RT-PCR showed that treated endothelial cells had a 3.5-fold increase with 20 µM troglitazone (Figure 2). This increase in AS mRNA could be inhibited by treatment with the transcriptional inhibitor
APPENDIX A: (Continued)

1-D-ribofuranosyl-benzimidazole (DRB) suggesting that the increase in steady-state AS mRNA levels was due to an increase in transcription rather than decreased AS mRNA turnover. These results also suggested that the increase in AS protein could be accounted for at the level of transcriptional regulation.

*Identification of a Putative PPRE in the Promoter of the AS Gene:* In order to account for the transcriptional regulation of AS expression by troglitazone, the AS promoter was examined using luciferase reporter gene constructs to identify regions regulated by this PPAR\(\gamma\) agonist. Previous work by others [26] and by us [27] has shown that three Sp1/3 elements in the proximal AS promoter are required for AS expression. Since PPAR\(\gamma\) agonists are known to mediate transcriptional effects through Sp1 elements [28, 29], we initially examined the involvement of the proximal promoter using a construct, p3ASP189, containing these three Sp1/3 elements in the first 189 bp of the AS promoter. However, transfection of the p3ASP189 construct into BAEC followed by treatment with troglitazone did not result in an increase in promoter activity (Figure 3A). Thus, the up-regulation of AS expression by troglitazone was not mediated by these Sp1 elements or other sequence elements located in the proximal promoter.

Based on these findings, the search to identify the element(s) involved in PPAR\(\gamma\) regulation was extended using a series of constructs containing increasing lengths of the AS promoter. Cells transfected with AS promoter constructs containing up to 2088 bp again showed no change in reporter gene activity in response to troglitazone treatment (data not shown). However, when a construct containing 2616 bp of the AS promoter was
transfected into BAEC, a significant increase in reporter gene expression was observed in response to treatment with troglitazone. The construct containing 2616 bp of the promoter was activated 2.7-fold by 20 μM troglitazone (Figure 3A). This comparative analysis of luciferase activity between treated and untreated transfections of the construct p3ASP2616 mapped the PPARγ responsive region from -2616 to -2088 bp upstream of the transcriptional start.

DNA sequence analysis identified a near consensus PPARγ response element (PPRE) from –2471 to –2458 bp (AGGTCAGGAGTTCA) in the p3ASP2616 construct. To verify the involvement of this element, comparative transient transfection assays were performed using a construct mutated (non-functional) in the putative PPRE and the wild-type construct. As shown in Figure 3B, mutation of the putative PPRE site in p3ASP2616 completely abolished the activating effects of troglitazone supporting the involvement of this PPRE (-2471 to -2458 bp) in the distal region of the AS promoter.

**PPARγ Binds to the AS PPRE:** To further confirm the involvement of the PPRE in AS promoter function, we investigated whether PPARγ binds to the putative AS PPRE. Electrophoretic mobility shift assays (EMSAs) were performed with oligonucleotides containing the putative sequence. Nuclear extracts from troglitazone and untreated BAEC were mixed with [³²P]-labeled AS PPRE oligonucleotides. As shown in Figure 4, troglitazone enhanced binding to the PPRE.
APPENDIX A: (Continued)

To demonstrate specificity, excess unlabeled PPREwt oligonucleotides were shown to compete, diminishing the signal of the shifted band. In contrast, addition of excess, unlabeled PPREmut oligonucleotide, with a mutation that should not allow binding and therefore should not compete with [32P]-labeled AS PPRE oligonucleotides, did not diminish the specific signal. These results were taken to further support the involvement of this distal PPRE in the AS promoter as the element that mediates the transcriptional upregulation by troglitazone.

Discussion

One mechanism by which PPARγ agonists provide cardiovascular benefits is by enhancing endothelial NO production [17]. Endothelium-derived NO is a potent chemical mediator with antiatherogenic properties, such as stimulation of vasorelaxation and repression of endothelial leukocyte adhesion molecules, platelet aggregation and smooth muscle cell proliferation [30-32]. Although troglitazone demonstrates vasodilator activities to lower blood pressure in diabetic patients, its precise mechanism is not well defined [20, 33, 34]. However, these studies suggest that troglitazone mediated direct effects on the vascular wall.

Until now, troglitazone was thought to promote endothelial NO production through up-regulation of eNOS protein expression [19] or activity [18], although the mechanism was not established. This report is the first demonstration that the PPARγ agonist, troglitazone, facilitates the production of vascular endothelial NO through the up-regulation of AS expression, the rate-limiting enzyme of the citrulline-NO cycle. The
increase in AS protein levels paralleled AS mRNA levels and the increased NO production. Since DRB, a transcriptional inhibitor, blocked the induction of AS expression by troglitazone, our results indicated that the increase in AS expression resulted from transcriptional regulation by this PPARγ agonist. Therefore, we identified a distal PPRE in the AS promoter that mediated the transcriptional effects of troglitazone on AS expression. To our knowledge, this is the first identification of a functional PPRE in the AS promoter.

These results further support our view that the coordinate regulation of endothelial AS expression and NO production is essential [5], and that physiologic or pharmacologic stimuli that promote or diminish endothelial function do so not only by affecting eNOS activity or expression, but also by affecting AS expression [5]. Moreover, the results in this report contribute new and additional insight as to how PPARγ agonists promote endothelial NO production through diverse mechanisms [18, 19, 35]. For example, 15d-PGJ2, a naturally occurring PPARγ ligand, increases hsp90 expression which promotes eNOS activation, while ciglitazone and rosiglitazone do not, yet still increase NO production [35]. In addition, 15d-PGJ2 and rosiglitazone increase binding of hsp90 to eNOS to promote NO production, while ciglitazone does not. Finally, both 15d-PGJ2 and rosiglitazone, but not ciglitazone, increase phosphorylation of eNOS at ser1177, which is linked to enhanced enzyme activity [35] and increased NO production. In this report, troglitazone was found to promote NO production through the up-regulation of AS expression. This would be in addition to its reported effect on eNOS where troglitazone was shown to up-regulate eNOS expression through a mechanism independent of PPARγ
activation [19], or where changes in eNOS phosphorylation correlated with an increase in eNOS activity rather than expression [18].

Overall, the findings of this report demonstrate that argininosuccinate synthase represents an additional and physiologically important step in the citrulline-NO cycle by which the TZD, troglitazone, promotes vascular endothelial function. Although troglitazone was withdrawn from the market because of its hepatic toxicity, the multiple mechanisms through which TZDs can improve insulin sensitivity, as well as NO-dependent vasodilatation, suggests that further studies with new TZD drugs may be warranted.

Acknowledgements

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APPENDIX A: (Continued)

References


APPENDIX A: (Continued)


APPENDIX A: (Continued)


APPENDIX A: (Continued)


Figure 1: The PPARγ agonist, troglitazone, stimulates endothelial NO production and AS protein expression. BAEC were treated with increasing concentrations of troglitazone as indicated for 24 hours. (A) NO was measured as nitrite produced/mg protein. (Nitrite is a stable reaction product of NO and molecular oxygen.) Results are expressed as relative levels of NO produced in control (no treatment) versus treated cells, and error bars represent the standard error of the mean. (B-C) Ten μg of whole cell lysate was loaded onto an SDS polyacrylamide gel and standard western blotting performed. Anti-AS (1:2500) was used to detect the amount of AS protein present. A representative western blot is shown in B, and relative spot density for AS protein, normalized against GAPDH and quantitated, is represented in C. These results are representative of three independent experiments and error bars represent the standard error of the mean.

Figure 2: Troglitazone induces transcription of AS mRNA. BAEC were untreated (U) or treated with 20 μM troglitazone plus or minus the transcriptional inhibitor DRB (50 μM) (T and T+D, respectively) for 24 hours. Total RNA was isolated, and AS mRNA was detected using real time quantitative RT-PCR. Results were normalized to 18S rRNA and represent the average ± the standard error of the mean.

Figure 3: Troglitazone induces a distal element in the AS promoter. (A) BAEC were transiently transfected with the proximal AS promoter construct, p3ASP189, or an
APPENDIX A: (Continued)

extended AS promoter construct, p3ASP2616, and treated with 20 µM troglitazone (Trog) for 24 hours. (B) BAEC were transiently transfected with the AS promoter constructs with wild type p3ASP2616 (W) or p3ASP2616PPREmut (M, represents mutated PPRE) and treated with 20 µM troglitazone (Trog) for 24 hours. All results are presented as relative luciferase activity units and represent the average ± the standard error of the mean of at least four experiments conducted in triplicate.

*Figure 4:* Troglitazone increases binding to the AS PPRE. (A) Electrophoretic mobility shift assays were performed using BAEC nuclear extracts prepared from untreated and troglitazone treated cells for 6 hours. Extracts were combined with an oligonucleotide probe containing the putative PPRE sequence of the AS promoter, and competed with either a 100-fold excess of cold wild-type or mutated oligonucleotide probe where indicated. Labeled arrow indicates position of PPARγ specific bands. (B) Relative density of PPARγ specific bands.
APPENDIX A: (Continued)

Figure A-1

A

![Graph showing fold NO produced vs. Troglitazone concentration](image)

B

![Image showing protein band with an arrow labeled AS](image)

C

![Graph showing relative spot density vs. Troglitazone concentration](image)
Figure A-2
APPENDIX A: (Continued)

A

![Bar chart with conditions: None - Untreated, Trog - Troglitazone]

B

![Bar chart with conditions: W - Wild Type Construct, M - Mutant Construct]

Figure A-3
APPENDIX A: (Continued)

A

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Figure A-4
ABOUT THE AUTHOR

Karen Davidowitz Corbin was born in San Juan, Puerto Rico. She obtained her Bachelor’s Degree in Nutrition and Food Science from Florida State University in 1997. She completed her Dietetic Internship at the James A. Haley Veterans Hospital in 1998. From there, she spent 5 years working as a clinical dietitian, certified diabetes educator and administrative director at The Heart and Vascular Institute of Florida as part of the LIFEHELP preventive medicine team and the CardioMAX heart failure program. Karen received her PhD in Molecular Medicine from the University of South Florida College of Medicine in 2008. Her short term goal is to conduct post-doctoral translational research related to nutrition and metabolic disorders utilizing both basic and genomic techniques. Her long term goals include contributing to nutrition genomic research and being a catalyst for moving the profession of dietetics securely into the genomic medicine era.