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Functional topology and regulation of endothelial nitric oxide synthase and associated caveolar components

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Functional Topology and Regulation of Endothelial Nitric Oxide Synthase and Associated Caveolar Components

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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Dedication

This dissertation is dedicated to my parents, Loretta and Eric Flam, who have always supported me in all my endeavors.
Acknowledgments

I would like to sincerely thank and express my gratitude to my mentors, Dr. Larry Solomonson and Dr. Duane Eichler, for allowing me to perform my doctoral dissertation research in their laboratory. They have always been supportive and have assisted me tremendously throughout the years. Thanks also go to my dissertation committee members: Dr. Craig Doupnik, Dr. John Hassell and Dr. Robert Potter, who have all provided valuable input into my dissertation and research. Dr. Richard Jove, a former member of my committee, also provided useful suggestions about my research.

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AS</td>
<td>argininosuccinate synthase</td>
</tr>
<tr>
<td>AL</td>
<td>argininosuccinate lyase</td>
</tr>
<tr>
<td>BAEC:</td>
<td>bovine aortic endothelial cells</td>
</tr>
<tr>
<td>BH₄</td>
<td>tetrahydrobiopterin</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>Hsp90</td>
<td>heat shock protein 90</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>O-GlcNAc</td>
<td>O-linked β-N-acetylglucosamine</td>
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ABSTRACT

The discovery of nitric oxide (NO) as the endothelial-derived relaxing factor has led to significant research on NO and the proteins involved in its function, generation, location and regulation. Synthesis of NO by blood vessel endothelial cells results from the enzymatic oxidation of arginine by endothelial nitric oxide synthase (eNOS) resulting in the formation of equimolar amounts of NO and citrulline. Citrulline is sequentially recycled to arginine by successive reactions involving the enzymes argininosuccinate synthase (AS) and argininosuccinate lyase (AL), respectively. eNOS activity has been shown to be regulated by post-translational modifications including dynamic phosphorylation on multiple serine/threonine and tyrosine residues and dynamic O-linked β-N-acetylglucosamine (O-GlcNAc) modifications on serine/threonine residues.

Previous studies showed that even though intracellular endothelial arginine levels range from 0.1 to 0.8 mM and the $K_m$ of eNOS for arginine is 3 µM, the addition of exogenous arginine caused an increase in NO production. To explain this “arginine paradox”, we hypothesize that there is a separate and distinct cellular source of arginine substrate directed to NO production and that this source is maintained through the regeneration of arginine via a citrulline-NO cycle. The presented research has provided the following evidence in support of this hypothesis:

- Citrulline stimulates NO production in an arginine-rich medium, without an increase in intracellular arginine.
- The enzymes of the citrulline-NO cycle, eNOS, AS and AL, co-fractionate with caveolin-1 in an endothelial cell caveolar membrane fraction.
In vitro interaction assays demonstrate protein-protein interactions between fusion tagged AS or AL with eNOS or caveolin-1.

Simultaneous monitoring of apparent citrulline and NO production demonstrates an efficient and essential coupling of the reactions of the citrulline-NO cycle.

Glucosamine treatment of endothelial cells results in increased NO production in the basal state and decreased NO production in the stimulated state.

Our findings demonstrate the enzymes of the citrulline-NO cycle, eNOS, AS and AL, are functionally associated, the reactions are efficiently coupled and enzyme activities are changed by post-translational modifications based on nutrient levels. These alterations ensure a constant and distinct source of arginine which is available for NO production to ensure vascular health.
Nitric oxide (NO) is a simple free radical once thought of only as an environmental toxin. However, in 1987, NO produced by endothelial cells was determined to be the endothelial-derived relaxing factor. Endothelial NO is necessary to regulate vascular tone, homeostasis, blood pressure and vascular remodeling (Naseem 2005).

NO is formed in vascular endothelial cells, platelets, macrophages and neuronal cells. Nitric oxide synthases (NOS) catalyze the reaction converting arginine to citrulline and NO in stoichiometric amounts (Fig. 1). In the endothelium, citrulline is recycled to arginine successively by the enzymes argininosuccinate synthase (AS) and argininosuccinate lyase (AL) with the conversion of citrulline to argininosuccinate as the rate-limiting step in the citrulline-NO cycle. There are three NOS isoforms encoded by separate genes, neuronal NOS (nNOS; NOS1), inducible NOS (iNOS, NOS2) and endothelial NOS (eNOS, NOS3), which share approximately 50% sequence homology (Feelisch et al. 1996). The NOS isoforms were named for the cells where they were originally discovered, but it was later found that many of the isoforms are expressed in other cell types, e.g. eNOS is also found in astrocytes. NOS requires the cosubstrates nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen and the cofactors flavin adenine dinucleotide, flavin mononucleotide, heme and tetrahydrobiopterin (BH₄) for activity and functionality. The constitutively active forms, nNOS and eNOS are membrane associated, require increased calcium concentrations for activation and bind the calcium-calmodulin complex in a reversible manner (Marletta 1994). iNOS, found in macrophages, neutrophils and vascular smooth muscle cells is induced by cytokines and is active at the lowest levels of calcium found in the cell. Both nNOS and eNOS produce intracellular NO at nanomolar concentrations while iNOS forms NO at micromolar concentrations (Blatter et al. 1995).
Figure 1. Citrulline-NO Cycle

The half-life of NO in aqueous solution is approximately 10 seconds. NO is lipophilic and acts on the heme iron center of soluble guanylyl cyclase (sGC) found in vascular smooth muscle cells and blood platelets (Ignarro 1990). NO activates sGC to convert guanosine triphosphate (GTP) to 3,5-cyclic guanosine monophosphate (cGMP), a second messenger. cGMP levels are increased up to several hundred-fold (Munzel et al. 2005). cGMP then causes a signaling cascade, and cytosolic calcium levels decrease in the smooth muscle cells because voltage-gated calcium channels are inhibited (Bolotina et al. 1994). Calcium is sequestered in the sarcoplasmic reticulum when protein kinases that phosphorylate sarcoplasmic reticulum proteins are activated (Cohen et al. 1999). When the cytosolic calcium levels drop, the phosphorylation of regulatory myosin light chains is affected, promoting smooth muscle relaxation (Horowitz et al. 1996). NO levels are adjusted in response to physiologic stimuli (e.g. bradykinin, shear stress and oxygen tension), intracellular and extracellular nutrient concentrations, genetic mutations in NOS gene sequences or changes in citrulline-NO cycle proteins.

Inadequate endothelial NO concentrations lead to endothelial dysfunction, which is observed in hypertension, diabetes, hypercholesterolemia, atherosclerosis and
cardiovascular disease (CVD) (Naseem 2005; Sase et al. 1997; Scherrer et al. 1994; Yoshizumi et al. 1993). CVD is an endemic disease in the Western world and is the fundamental cause of many deaths (Ignarro et al. 1987a; Ignarro et al. 1987b; Palmer et al. 1987). Increasingly, CVD is appearing in Japan, India and Eastern Europe due to increased Westernization (Naseem 2005). CVD incidence is increased by factors such as hypercholesterolemia, hyperglycemia, obesity, high blood pressure and smoking. However, the underlying pathology is atherosclerosis, the deposition of plaques containing cholesterol and lipids on the damaged endothelial cell layer in the arteries and other large vessels (Naseem 2005; Widlansky et al. 2003). Inadequate synthesis and increased NO inactivation (NO insufficiency) leading to endothelial dysfunction is the earliest stage of atherosclerosis (Voetsch et al. 2004). The first phase of atherosclerosis is alteration in endothelial cell phenotype causing dysfunction of the endothelial cells lining the vasculature. These changes occur because of high blood pressure, high glucose levels (caused by diabetes) and high lipid concentrations. Once there is an impaired endothelial section, endothelial cells express adhesion molecules on their cell surface and monocytes attach to the damaged cells (Quyyumi 1998). These monocytes differentiate into macrophages which destroy the damaged endothelial cells. In this scenario, macrophages fail to return to the circulation and accumulate lipids. As a consequence, smooth muscle cells at these sites change their phenotypic characteristics, becoming less contractile. This new phenotype secretes collagen, and participates in the formation of a fibrous mass, or plaque. Plaque formation eventually may reduce or block blood flow in localized regions. Loss of oxygen and nutritional transport occurs and the affected cells become necrotic. Local angiogenesis replaces the dead endothelial cells in the blood vessels, further enhancing plaque formation. If the fibrous mass is thin, an actual rupture of the vessel may occur. At this point, blood platelets would be activated to form a clot to prevent bleeding and loss of vessel integrity. If these events occur in the coronary artery, death can ensue since the heart is no longer supplied with oxygenated blood (Naseem 2005). In individuals with uncompromised cardiovascular systems, NO inhibits smooth muscle proliferation to prevent overproduction of the extracellular matrix proteins leading to atherosclerosis (Lusis 2000; Napoli et al. 2006).
Lifestyle and diet influence the risk for CVD. Obesity, often associated with a sedentary lifestyle and/or overeating, leads to endothelial dysfunction and oxidative stress. In obesity, there are high circulating levels of tumor necrosis factor alpha (TNFα), which can cause circulating monocytes to adhere to vascular endothelial cells eventually leading to atherosclerotic lesions. In response to increased circulating levels of TNFα, adiponectin circulating levels are decreased in obese animals and humans (Chen et al. 2003). Full length adiponectin prevents the expression of certain adhesion molecules like VCAM (due to increased TNFα levels) on the endothelial cell surface, preventing circulating monocyte adhesion, an early hallmark of atherosclerosis (Goldstein et al. 2004). Adiponectin also regulates endothelial NO production (Hattori et al. 2003). Both the full length and proteolytic cleavage product, globular adiponectin, were shown to prevent atherosclerosis in apolipoprotein E knockout mice (Goldstein & Scalia 2004). Hattori et al. determined that human umbilical vein endothelial cells treated with globular adiponectin had increased eNOS mRNA and protein expression and that globular adiponectin treatment caused an increase in phosphorylation of eNOS increasing NO levels (Hattori et al. 2003). However, administration of globular adiponectin along with TNFα, which is known to decrease eNOS mRNA stability (Anderson et al. 2004), failed to prevent the accelerated eNOS mRNA degradation caused by TNFα (Hattori et al. 2003). Using human aortic endothelial cells, Tan et al. determined that adiponectin increases NO production in these cells (Tan et al. 2004). More recently, Xi et al. determined that eNOS phosphorylation caused by globular adiponectin produced an association between eNOS, heat shock protein 90 (hsp90) and protein kinase B (Akt) resulting in increased NO production (Xi et al. 2005). When hsp90 association with eNOS was inhibited in bovine aortic endothelial cells (BAEC) treated with globular adiponectin, there was a decrease in endothelial NO production (Xi et al. 2005). When aortic rings were incubated with L-NAME, an eNOS inhibitor, globular adiponectin had no effect on vasodilation (Xi et al. 2005).

Hyperglycemia contributes to endothelial dysfunction and at least three mechanisms have been proposed to account for the inhibition of endothelial NO production by elevated serum glucose (Buse 2006; Ding et al. 2004; Ho et al. 1999; Wu
et al. 2001b). It has been postulated that elevated serum glucose as well as glutamine and glucosamine, may inhibit NO production by promoting hexosamine synthesis in endothelial cells.

Figure 2. Hexosamine Biosynthetic Pathway

In the hexosamine biosynthetic pathway (Fig. 2), UDP-N-acetylglucosamine (UDP-GlcNAc) is produced and used as the donor sugar for O-linked β-N-acetylglucosamine (O-GlcNAc, Appendix D) protein modifications (Wells et al. 2003). The enzyme O-GlcNAc transferase transfers O-GlcNAc from UDP-GlcNAc to serine or threonine residues on proteins (Haltiwanger et al. 1992) while O-GlcNAcase removes O-GlcNAc from serine or threonine residues (Dong et al. 1994). O-GlcNAcase activity is inhibited by O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-N-phenylcarbamate (PUGNAc, Appendix D). Glutamine and fructose 6-phosphate are combined to form glucosamine 6-phosphate which is eventually converted to UDP-GlcNAc. Glucosamine (Appendix D), synthesized from fructose-6-phosphate and glutamine, enters the hexosamine biosynthetic pathway past the glucosamine:fructose-6-phosphate amidotransferase (GFAT) rate limiting step and was shown to increase O-GlcNAc modifications on eNOS protein near the protein kinase B phosphorylation site (Du et al. 2001). Wu et al. demonstrated that endothelial cells cultured in glucosamine had decreased levels of NO production which was attributed to decreased cellular cofactor (NADPH) availability (Wu et al. 2001a; Wu et al. 2002). They also demonstrated that high glucose and glutamine concentrations decrease endothelial NO production and this
inhibition was reversed by incubation with the GFAT inhibitor, 6-diazo-5-oxo-L-norleucine (DON, Appendix D). Elevated concentrations of glucose and glucosamine were both shown to inhibit NO production in cultured endothelial cells (Buse 2006). Glucose transporters on the cell surface transport glucose or glucosamine into the cell, which can increase O-GlcNAc modifications found on proteins under conditions of high glucose, such as in diabetes (Akimoto et al. 2005). O-GlcNAc modifications of certain proteins may cause a desensitization of glucose transport by causing a change in the movement of glucose transporters to the cell surface for glucose uptake, possibly leading to insulin resistance (McClain 2002; McClain et al. 2002; Walgren et al. 2003). Dynamic O-GlcNAc modifications also regulate the activities of eNOS, Sp1 and calmodulin and typically occur in response to changes in the cellular environment in a manner similar to phosphorylation/dephosphorylation and may involve substitution of the same residues (serine/threonine) (Hart 1997; Jackson et al. 1988b; Majumdar et al. 2006; Slawson et al. 2003; Whelan et al. 2003; Zachara et al. 2006). Leptin gene transcription, important in insulin-resistance and obesity, is modulated by O-GlcNAcase and levels of UDP-GlcNAc (Wang et al. 1998).

There are many transcription factors that also contain O-GlcNAc modifications, with Sp1 being the most well characterized (Jackson & Tjian 1988b). Sp1 is modified by phosphorylation and O-GlcNAc modification (Jackson et al. 1993; Jackson & Tjian 1988b). These modifications change Sp1’s association with transcription binding sites in gene promoter regions causing changes in mRNA and protein expression. AS, another essential protein for NO production and endothelial survival contains three Sp1 transcription binding sites within the human AS promoter (Anderson et al. 1991). In Caco-2 cells, a human intestinal cell line, glutamine and glucosamine were shown to increase O-GlcNAc modification of Sp1 causing greater nuclear translocation. There was greater Sp1 binding to the AS promoter initiating increased transcription (Brasse-Lagnel et al. 2003). Nandi et al. (Nandi et al. 2006) demonstrated a technique to locate O-GlcNAc modified proteins on a global scale for proteomic analysis. The authors confirmed O-GlcNAc modifications on many proteins while increasing the known
number of O-GlcNAc modified proteins to over 200 including the determination that AS can be O-GlcNAc modified (Nandi et al. 2006).

A third essential protein for endothelial NO production, AL, contains one Sp1 and one nuclear factor-Y (NF-Y) transcription factor binding site in the rat AL promoter (Matsubasa et al. 1994; Takiguchi et al. 1995). Examination of the NF-Y sequence with the YinOYang prediction server shows many potential O-GlcNAc modification sites (Blom et al. 1999). This prediction server contains O-GlcNAc modified protein amino acid sequences in its database and attempts to predict O-GlcNAc attachment sites in proteins using these data (Love et al. 2005). It is possible, but has not been demonstrated, that O-GlcNAc modification of NF-Y causes a change in the binding of this transcription factor to the AL promoter and changes AL gene transcription.

Genetic polymorphisms in the eNOS gene which spans 26 exons may result in decreased NO production. One polymorphism is linked to a reduction in promoter activity. Another polymorphism which causes a change in the primary structure of the protein causes a reduction in NO production in healthy individuals. Other polymorphisms can cause changes in protein stability, post-translational modifications, intracellular distribution or cofactor association (Naseem 2005).

Endothelial NOS activity may be regulated by a number of mechanisms including lipidation, calcium-calmodulin interaction, protein-protein interactions, phosphorylation, O-linked glycosylation and substrate/cofactor availability (Schulz et al. 2005). eNOS regulation involves reversible phosphorylation on serine, threonine and tyrosine that affects the activity and subcellular localization of eNOS (Fulton et al. 2005; Michel et al. 1993). This phosphorylation/dephosphorylation causes changes in shear stress and contributes to vascular complications from CVD and type 2 diabetes. Depending on the type of stimulus, different residues in eNOS can be phosphorylated resulting in either an up- or down-regulation of eNOS activity. Phosphorylation by PKB, protein kinase G and AMP-activated protein kinase, at serine 1179 (bovine) in eNOS results in an increase in enzymatic activity by increasing electron flux through the eNOS reductase domain (Butt et al. 2000; Chen et al. 1999; Dimmeler et al. 1999; Fulton et al. 1999; Gallis et al. 1999; McCabe et al. 2000). Phosphorylation of serine 635 activates eNOS and phosphorylation...
of serine 617 increases eNOS calcium-calmodulin sensitivity leading to increased activity (Michell et al. 2002). However, phosphorylation of threonine 497 by PKC inhibits eNOS activity by decreasing eNOS calcium-calmodulin sensitivity (Fleming et al. 2001; Schulz et al. 2005). However, Lin et al. demonstrated that threonine 497 dephosphorylation in eNOS caused an increase in both NO and superoxide production and that the production of these products was balanced by eNOS accessory proteins and protein phosphorylation (Lin et al. 2003). Serine 116 phosphorylation decreases eNOS activity (Corson et al. 1996; Kou et al. 2002). Tyrosine 83 phosphorylation by Src kinase was associated with a three-fold increase in basal NO release from transfected COS-7 cells (Fulton et al. 2005).

NOS is regulated by and targeted to different subcellular fractions by post-translational modifications. For example, eNOS is acylated — either myristoylated, palmitoylated or both — targeting it to the cell membrane (Busconi et al. 1993; Liu et al. 1994; Pollock et al. 1992). Palmitoylation is a reversible modification which can serve as a mechanism for changing the way a protein is regulated under different physiologic states (Chen et al. 2001). For instance, COS-7 cells were transfected with palmitoylation-deficient eNOS or myristoylation- and palmitoylation-deficient eNOS (Shaul et al. 1996). There was a threefold increase in myristoylated eNOS in caveolae in cells transfected with the palmitoylation-deficient eNOS, and a tenfold increase in palmitoylated and myristoylated eNOS in caveolae in cells transfected with wild-type eNOS, when compared to cells transfected with the myristoylation and palmitoylation-deficient eNOS. In cells treated with bradykinin, Robinson et al. showed that this agonist promoted the loss of eNOS palmitoylation causing the enzyme to change its subcellular localization and translocate from the membrane to the cytosol (Robinson et al. 1995). Also, in response to certain agonists, eNOS can be recovered in the soluble supernatant fraction from cell lysates (Michel et al. 1993). Targeting of eNOS to membranes was further investigated by Shaul et al. who found that palmitoylated eNOS is targeted to a specific invaginated region of the plasma membrane (Shaul et al. 1996). This region is called the caveola, meaning “small cave” (Yamada 1955). Caveolae are characterized by high cholesterol concentration resulting in a low buoyant density and enrichment in glycosphingolipids (Pike et al. 2002; Shaul et al. 1998; Smart et al. 1995). More than 50
signaling molecules are localized to caveolae which are found in abundance in such cells as adipocytes, fibroblasts and endothelial cells (Schulz et al. 2005). Endothelial cell caveolae contain the structural protein caveolin-1, an integral membrane protein. Caveolin-1 forms oligomeric complexes of up to 15 molecules with a molecular mass of ~350 kDa (Spisni et al. 2005) and binds cholesterol in a 1:1 ratio (Murata et al. 1995). Approximately 15% of the endothelial cell volume is comprised of caveolae and ~95% of the plasma membrane vesicles are caveolae (Wang et al. 2005). The N- and C-terminal domains of caveolin-1 interact with eNOS and inhibit its catalytic activity by interfering with electron transport from the reductase to the oxygenase domain of eNOS (Garcia-Cardena et al. 1997; Ghosh et al. 1998; Ju et al. 1997; Michel et al. 1997a; Michel et al. 1997b). Gonzalez et al. (Gonzalez et al. 2004) described experiments using small interfering RNAs to knockdown caveolin-1 protein expression in endothelial cells. They reported that although caveolin-1 protein levels were decreased by approximately 90%, many signaling molecules, including eNOS, were still targeted to the endothelial cell membrane.

Protein-protein interactions also modulate eNOS activity. Some of the proteins shown to interact with eNOS are caveolin-1, eNOS interacting protein (NOSIP), eNOS traffic inducer (NOSTRIN), hsp90 a cytosolic chaperone protein, AS and AL (Dedio et al. 2001; Flam et al. 2001; Garcia-Cardena et al. 1997; Ju et al. 1997; Pritchard et al. 2001). When endothelial cells are stimulated with calcium agonists or by shear stress, there is a displacement of eNOS from caveolin-1 in the caveolae facilitated by a calcium-calmodulin complex. Following the release of eNOS from caveolin-1, eNOS binds to hsp90. Hsp90 also recruits PKB and prevents dephosphorylation of PKB by protein phosphatase 2A (Schulz et al. 2005). This action can help increase the phosphorylation of eNOS at serine 1177 (human). In COS-7 cells transfected with eNOS and carboxyl terminus of Hsp70-interacting protein (CHIP), a co-chaperone protein of hsp90, Jiang et al. showed that soluble eNOS was segregated to an insoluble and inactive cellular compartment (Jiang et al. 2003). The authors concluded that CHIP was incorporated into the eNOS-hsp90 complex. To complement these observations, basal eNOS activity measured in lung endothelial cell lysates from CHIP -/- mice was significantly greater
than from CHIP +/+ cell lysates (Jiang et al. 2003). To inhibit NO synthesis, NOSIP, binds to the oxygenase domain of eNOS and causes translocation of eNOS from the plasma membrane to intracellular sites (Dedio et al. 2001). Similarly, overexpression of NOSTRIN causes a decrease in eNOS activity due to its translocation away from the plasma membrane (Oess et al. 2006; Zimmermann et al. 2002).

Cofactor availability can change NO production. A reduction in the levels of the pterin, BH₄, an essential eNOS cofactor, causes a decrease in NO production. Werner et al. reported that suboptimal concentrations of BH₄ cause eNOS to function as a monomer in the uncoupled state, converting arginine to superoxide rather than NO (Werner et al. 1996). These superoxide radicals can react with NO forming peroxynitrites, which further reduce NO levels causing inactivation (Stroes et al. 1998). These peroxynitrites are strong oxidants that can be protonated and, in high concentration, can undergo either heterolytic or hemolytic cleavage forming damaging free radicals (Brovkovych et al. 1999). This leads to oxidative stress and damage to eNOS (Zou et al. 2002). Supplementation with sepiapterin, a BH₄ precursor, or BH₄ alone can prevent superoxide generation (Werner-Felmayer et al. 1993). It is presumed that in vascular endothelial dysfunction, most bioavailable NO is inactivated by its reaction with superoxide (Naseem 2005). As a result, NO levels are reduced, leading to vasoconstriction, blood pressure increases and eventually CVD if left unresolved (Malinski 2005).

Endothelial NOS substrate availability also regulates endothelial NO production. Arginine, first isolated from lupin seedlings, is classified as a semi-essential amino acid because at times the body is unable to synthesize enough for cellular processes depending on subject age, disease or injury (Morris 2004). In 1988, arginine was determined to be the physiologic precursor of NO (Palmer et al. 1988). Average dietary intake of arginine in the United States is 5.4 g/day (Visak 1986). However, due to high arginase activity in the small intestine, approximately 40% of the ingested arginine is degraded during absorption (Flynn et al. 2002). Intravenous arginine administration is an effective method of introducing arginine to extrahepatic tissues, like the endothelium (Flynn et al. 2002). Mammalian plasma arginine levels are 80 ± 20 µM (Cynober 2002). Arginine can be converted to other metabolites by enzymes in mammalian cells such as nitric oxide
synthases, arginases, arginine:glycine amidino-transferase and arginine decarboxylase (Morris 2004). Most arginine is synthesized in the intestinal-renal axis pathway by epithelial cells in the small intestine and proximal tubular cells of the kidney which produce arginine from glutamine and glutamate (Morris 2002; Morris 1992). Therefore renal function impairment can affect the body’s ability to synthesize arginine leading to a need for increased arginine intake. Arginase 2 which converts arginine to ornithine and urea is expressed in endothelial cells (Wu et al. 1998). The $K_m$ of arginase for arginine is 2-20 mM while the $K_m$ of NOS for arginine is 2-20 µM (Davis et al. 1998).

Extracellular arginine is transported into endothelial cells mainly by the system $y^+$ transporter, a high affinity, sodium-independent transporter that transports basic amino acids like arginine, lysine, histidine and ornithine (Closs et al. 2000). This transporter is responsible for ~70-90% of arginine uptake in endothelial cells (Zharikov et al. 2001). The CAT1 transporter, a system $y^+$ transporter, mediates arginine uptake in porcine aortic endothelial cells and is responsible for 60-80% of arginine uptake (McDonald et al. 1997). However, there are other arginine transport systems including L, $B^{0,+}$ (sodium dependent) and $b^{0,+}$ (Bae et al. 2005).

Citrulline, found in high concentrations in watermelon, obtains its name from the Latin for watermelon, Citrullus vulgaris. Even though citrulline is a non-essential amino acid and is not used in protein synthesis, it is an important amino acid, especially in the citrulline-NO cycle (Curis et al. 2005). Mammalian plasma citrulline levels are 38 ± 8 µM (Cynober 2002).

For those individuals with cardiovascular risk factors like hypercholesterolemia, hypertension, diabetes, obesity and coronary artery disease, arginine administration has sometimes proved effective in improving endothelial function (Bode-Boger 2006; Bode-Boger et al. 2003; Loscalzo 2004; Maxwell et al. 2001; Preli et al. 2002; Wu et al. 2000; Wu & Meininger 2002). Although arginine is not toxic, high oral doses may result in nausea and gastrointestinal distress due to rapid NO production in the gut or poor absorption of other basic amino acids (Cooke et al. 2000). More recently citrulline has also been used to treat endothelial dysfunction in an effort to increase endothelial NO production (Curis et al. 2005; Hayashi et al. 2005). It may be a better alternative than
arginine since it is used in less cellular processes, the main function in endothelial cells is to be recycled to arginine for endothelial NO production.

Glutamine also plays a role in regulating endothelial NO synthesis. Hecker et al. and others demonstrated a decrease in NO production in cultured endothelial cells in the presence of increasing concentration of glutamine although each group suggested different mechanisms (Arnal et al. 1995; Hecker et al. 1990a; Meininger et al. 1997; Su et al. 1995; Wu et al. 2001a; Wu et al. 1993). It is thought that glutamine may inhibit AS leading to a decrease in recycling of citrulline to arginine and consequently a decrease in endothelial NO production. Glutamine does not change eNOS expression or essential cofactor concentrations (Wu et al. 2001b). Rats that were fed a diet rich in glutamine showed a decrease in NO production in vivo (Houdijk et al. 1998). Glutamine can be metabolized to glucosamine and may decrease intracellular NADPH levels, leading to a decrease in NOS activity and NO production (Wu et al. 2001a). As mentioned previously, glutamine enters the hexosamine biosynthetic pathway ultimately leading to increased intracellular UDP-GlcNAc levels. These increased UDP-GlcNAc concentrations lead to O-GlcNAc protein modification and for eNOS, decreased enzyme activity (Du et al. 2001).

Vane and others determined that arginine was essential for NO synthesis and that the enzymes of the citrulline-NO cycle, eNOS, AS and AL were essential to ensure regeneration of the arginine substrate for endothelial NO production (Flam et al. 2001; Hecker et al. 1990b; Wu & Meininger 1993). They showed that even though intracellular endothelial arginine levels are on the order of 0.1-0.8 mM and the \( K_m \) of eNOS for arginine is \(~3 \mu M\), endothelial NO production is enhanced by the addition of exogenous arginine (Hardy et al. 2002; Hecker et al. 1990b; Mitchell et al. 1990). Endothelial NO is an important component in maintaining the health of the cardiovascular system. As outlined in this introduction, the ability to control and optimize the production of NO is critical to the reduction of morbidity and mortality due to cardiac failure. Research to determine the location, function and regulation of eNOS and other associated caveolar components which support endothelial NO production is vital to this goal. The following
research has been focused in this area and the results are in the four manuscripts which form the body of my dissertation. The major results of this body of work are:

· Citrulline stimulates NO production in an arginine-rich medium, without affecting intracellular arginine levels.
· The enzymes of the citrulline-NO cycle, eNOS, AS and AL, co-fractionate with caveolin-1 in an endothelial cell caveolar membrane fraction.
· *In vitro* interaction assays demonstrate protein-protein interactions between fusion tagged AS and AL with eNOS and caveolin-1.
· Simultaneous monitoring of apparent citrulline and NO production demonstrates an efficient and essential coupling of the reactions of the citrulline-NO cycle.
· Glucosamine treatment of endothelial cells results in increased NO production in the basal state and decreased NO production in the stimulated state.
Paper I:
Caveolar Localization of Arginine Regeneration Enzymes, Argininosuccinate Synthase and Lyase, with Endothelial Nitric Oxide Synthase

by

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Abstract

Although normal intracellular levels of arginine are well above the $K_m$, and should be sufficient to saturate nitric oxide synthase in vascular endothelial cells, nitric oxide production can, nonetheless, be stimulated by exogenous arginine. This phenomenon, termed the “arginine paradox”, has suggested the existence of a separate pool of arginine directed to nitric oxide synthesis. In this study, we demonstrate that exogenous citrulline was as effective as exogenous arginine in stimulating nitric oxide production, and that citrulline in the presence of excess intracellular and extracellular arginine further enhanced bradykinin stimulated endothelial nitric oxide production. The enhancement of nitric oxide production by exogenous citrulline could therefore be attributed to the capacity of vascular endothelial cells to efficiently regenerate arginine from citrulline. However, the regeneration of arginine did not affect the bulk intracellular arginine levels. This finding not only supports the proposal for a unique pool of arginine, but also suggested channeling of substrates that would require a functional association between nitric oxide production and arginine regeneration. To support this proposal, we showed that nitric oxide synthase, and the enzymes involved in arginine regeneration, argininosuccinate synthase and argininosuccinate lyase, co-fractionated with plasmalemmal caveolae, a subcompartment of the plasma membrane. Overall, the results from this study strongly support the proposal for a separate pool of arginine for nitric oxide production that is defined by the cellular co-localization of enzymes involved in nitric oxide production and the regeneration of arginine.

Key Words: endothelial nitric oxide synthase, citrulline, argininosuccinate synthase, argininosuccinate lyase
Introduction

The body’s vascular endothelial cells constitute a large potential reservoir for regulation of blood vessel tone through the vasodilatory effects of endothelial nitric oxide (NO). Impaired production of endothelial nitric oxide has been associated with hypertension, heart failure, hypercholesterolemia, atherosclerosis and diabetes. Circulating effectors, such as bradykinin, bind to receptors on the luminal surface of endothelial cells signaling the transient release of nitric oxide to the adjacent smooth muscle layer, resulting in “relaxation” of the vessel wall. To affect endothelial nitric oxide production, the enzyme that catalyzes the formation of nitric oxide in endothelial cells, endothelial nitric oxide synthase (eNOS), is subject to multiple modes of regulation, including Ca\(^{2+}\)-calmodulin binding, reversible phosphorylation and palmitoylation, substrate and cofactor availability, dimerization of enzyme subunits, and intracellular translocation (Sase & Michel 1997).

In cultured endothelial cells, intracellular arginine concentrations have been reported to range from 0.1 to 0.8 mM (Baydoun et al. 1990; Block et al. 1995; Gold et al. 1989; Harrison 1997; Hecker et al. 1990b; Mitchell et al. 1990). At these concentrations of substrate, eNOS would be operating at or close to maximal velocity since reported \(K_m\) values for arginine are less than 10 µM (Harrison 1997). However, a number of studies have shown that nitric oxide production by vascular endothelial cells under physiological conditions can be increased by extracellular arginine, despite intracellular arginine concentrations that should be “saturating” (Aisaka et al. 1989; Cooke et al. 1991; Eddahibi et al. 1992; Rossitch et al. 1991). These results suggest that the source of arginine utilized by eNOS is distinct from bulk cellular arginine pools. To explain these findings and to identify the source of arginine utilized by eNOS, McDonald et al. showed that the CAT1 transporter, responsible for 60-80% of total carrier-mediated arginine transport into endothelial cells, co-localizes in the plasma membrane in caveolae with endothelial nitric oxide synthase (Greene et al. 1993; McDonald et al. 1997a; McDonald et al. 1997b). Thus, they proposed that the arginine utilized by eNOS, at least in part, may be maintained by the CAT1 transporter accessing serum arginine (~90 µM).
An alternative source of arginine has also been suggested to result from the recycling of citrulline. Hecker et al. (Hecker et al. 1990b) initially demonstrated that citrulline, produced in the conversion of arginine to NO, can be recycled to arginine in cultured endothelial cells. Other cell types have subsequently been found to have the capacity to regenerate arginine from citrulline utilizing two of the urea cycle enzymes, argininosuccinate synthase and argininosuccinate lyase (Shuttleworth et al. 1995; Xie et al. 2000). In neural tissue, histochemical staining for the localization of nitric oxide synthase demonstrated that argininosuccinate synthase, one of the enzymes involved in arginine regeneration is co-localized with nitric oxide synthase (Arnt-Ramos et al. 1992; Shuttleworth et al. 1995).

A linkage between arginine regeneration (citrulline-arginine cycle) and NO production has also been established for cells that contain the inducible form of nitric oxide synthase (iNOS). Such cells have a high and sustained demand for arginine owing to the large and Ca\(^{2+}\)-independent increase in activity following induction of iNOS synthesis by inducers such as bacterial lipopolysaccharide (LPS) and certain cytokines. Major lines of evidence for a linkage between the arginine regeneration and immunostimulant-induced nitric oxide production include a demonstration of a co-induction of iNOS and argininosuccinate synthase in a murine macrophage cell line (Nussler et al. 1994) and enhancement of immunostimulant-induced NO production in vascular smooth muscle cells following transfection with argininosuccinate synthase (Xie et al. 1997).

Several reports have further indicated a possible role for arginine regeneration in the transient, Ca\(^{2+}\)-dependent, production of nitric oxide in vascular endothelial cells, but the evidence has been less direct. For example, Su and Block showed that during hypoxia, both nitric oxide production and arginine regeneration in endothelial cells were inhibited (Su & Block 1995); however, in this case the effect of hypoxia on nitric oxide production could also be explained on the basis of the $K_m$ for oxygen use by eNOS (Rengasamy et al. 1996). Most significant, however, was a report where two infants with a deficiency in argininosuccinate lyase, who could therefore not regenerate arginine, were shown to be hypertensive. Upon infusion of L-arginine, these patient’s blood pressure
decreased, supporting the critical role for arginine regeneration (citrulline-arginine cycle) in the regulation of systemic blood pressure (Fakler et al. 1995).

Although previous reports support the role of the citrulline-arginine cycle in the regeneration of arginine for endothelial nitric oxide production, there has heretofore been no strong evidence that the arginine generation system is directly coupled to the receptor-mediated stimulation of nitric oxide production in vascular endothelial cells. In this report, we present evidence that arginine regeneration from citrulline is rate-limiting for bradykinin-mediated nitric oxide production, and that the enzymes involved in recycling citrulline to arginine are co-localized in discrete cellular domains (caveolae) with nitric oxide synthase. The localization of the arginine regeneration enzymes with caveolae and the synergistic response to citrulline and arginine supports the hypothesis that the citrulline-arginine cycle plays an important, and possibly essential, role in the production of nitric oxide by vascular endothelial cells.
Experimental Procedures

**Materials** – Dulbecco’s modified Eagle’s medium (DMEM), antibiotic/antimycotic, and gentamycin were obtained from Cellgro by Mediatech. Fetal bovine serum was purchased from Hyclone Labs. Bovine aortic endothelial cells (BAEC) were isolated from bovine aorta and were also obtained from a commercially available source (Clonetics). Percoll, Tricine, L-citrulline, and L-aspartate were acquired from Sigma. Sucrose was from BRL, Life Technologies Inc. OptiPrep™ was purchased from Gibco BRL. Broad range prestained protein markers were from New England BioLabs. Antibodies used for immunoblotting were: anti-caveolin-1 IgG (Transduction Laboratories), anti-NOS3 (c-20) IgG (Santa Cruz Biotechnology), anti-ecNOS IgG (Transduction Laboratories), anti-argininosuccinate synthase IgG (AnaSpec Inc.), anti-argininosuccinate lyase IgG (AnaSpec Inc.), anti-GM130 (Golgi Matrix Protein of 130 kDa, Transduction Laboratories), goat anti-rabbit IgG horseradish peroxidase (HRP) conjugated (Santa Cruz Biotechnology, Jackson ImmunoResearch Laboratories) and goat anti-mouse IgG horseradish peroxidase (HRP) conjugated (Jackson ImmunoResearch Laboratories).

**Preparation of Antibodies against Argininosuccinate Synthase (AS) and Argininosuccinate Lyase (AL) Peptide Sequences** – A peptide region corresponding to amino acid residues 41-55 of the bovine sequence for AS and a peptide region corresponding to residues 89-103 of the human sequence for AL were used to prepare the AS and AL antibodies, respectively. Both antibodies and peptides were prepared by AnaSpec, Inc. of San Jose, CA. Antibody specificity was determined by performing a peptide neutralization procedure where the antibody was neutralized by preincubation with excess antigen (peptide). Antibody and 5x excess of corresponding peptide (by weight) were incubated overnight at 4°C on a rotator. This mixture was then added to blocking buffer and a standard Western blot procedure was performed.

**Growth and Stimulation of Endothelial Cells** – BAEC were grown to confluence (6-well cluster dishes; 9.6 cm²/well) in high-glucose DMEM containing 10% fetal bovine serum, penicillin, streptomycin, gentamycin and amphotericin B. Cells were used 3 days after confluence.
Nitric Oxide Production Assay – Production of nitric oxide by endothelial cells was monitored by measuring the stable nitric oxide degradation product, nitrite, in the culture media. Nitrite content in the media was assayed using a fluorometric method (Misko et al. 1993).

HPLC Analysis of PITC-Derivatized Amino Acids from Endothelial Cell Lysates – Cells were lysed in 90% methanol, and the soluble fraction derivatized by PITC (phenylisothiocyanate). HPLC analysis was carried out on a Beckman HPLC System equipped with two model 160 pumps and controller, and a Supelcosil LC-18-DB column (5 µm, 4.6 mm x 250 mm) (Supelco) maintained at 45°C with a circulating water bath. Separation was achieved by controlling the solvent composition. Solvent A was 50 mM ammonium acetate, with the pH adjusted to 6.8 with ammonium hydroxide. Solvent B was 100 mM ammonium acetate: methanol (30:70), with the pH adjusted to 6.8 using acetic acid. The elution program consisted of the following: (a) initial conditions, flow rate equal to 1.0 ml/min, 5% B, gradient curve value 6 (concave gradient); (b) starting at time zero, concave gradient to 10% B over 12 min; (c) at 12 min, concave gradient to 30% B over 48 min; (d) at 60 min, concave gradient to 60% B over 7 min; (e) at 67 min, concave gradient to 5% B over 3 min; (f) at 70 min, return to initial conditions for 20 min. The eluate was monitored at 254 nm with a Beckman model 160 absorbance detector, and the PITC-amino acids were quantified by the integral values of their peaks.

Caveolar Fractionation – Caveolar preparations were carried out using approximately 5 x 10^8 BAEC. Two different caveolar fractionation protocols were followed. The first was a detergent-free, non-alkaline protocol reported by Smart et al. (Smart et al. 1995) involving multiple gradient fractionations under neutral pH conditions. Media was removed and the cell culture flasks were washed with Buffer A (250 mM sucrose, 1 mM EDTA, 20 mM Tricine, pH 7.8, 1 mM L-citrulline, and 1 mM L-aspartate) and the cells were collected by scraping into 10 ml of Buffer A and by centrifugation at 1400g for 5 min. The cell pellet was resuspended in 1.0 ml of Buffer A and homogenized using a glass-Teflon homogenizer, and the suspension was centrifuged at 1000g for 10 min. The pellet was resuspended in 1.0 ml of Buffer A and re-homogenized as described previously. The membrane fraction was collected again by
centrifugation and the supernatant pooled with the first and designated as the post-nuclear supernatant (PNS). The PNS fraction was layered on top of 30% Percoll (in Buffer A), and centrifuged at 84,000×g for 30 min. A visible band representing the plasma membrane was removed and sonicated, then mixed with Buffer C (125 mM sucrose, 3 mM EDTA, 60 mM Tricine, pH 7.8, 1 mM L-citrulline, and 1 mM L-aspartate in 50% OptiPrep). A 10-20% gradient of OptiPrep in Buffer C diluted with Buffer A was layered on top of the sample and centrifuged at 53,000×g for 90 min. The top 5 ml of the gradient were collected and 2 ml of 5% OptiPrep in Buffer C diluted with Buffer A were overlayed and the samples were again centrifuged. Fractions were collected and analyzed.

The second procedure described was carried out according to Song et al. (Song et al. 1996) using only a single discontinuous gradient of sucrose in Mes-buffered saline (MBS; 25 mM Mes, pH 6.5, 150 mM sodium chloride, 1 mM L-citrulline, 1 mM L-aspartate) containing 250 mM sodium carbonate. BAEC were washed twice with cold phosphate-buffered saline (PBS), scraped into approximately 15 ml of PBS and pelleted at 1400×g for 5 min. The supernatant was aspirated and the cell pellet was resuspended in 1 ml of freshly prepared 500 mM sodium carbonate buffer, pH 11, containing 1 mM L-citrulline and 1 mM L-aspartate. The cell suspension was homogenized (10 strokes) using a Dounce homogenizer, then further disrupted by a Polytron tissue grinder (three bursts) followed by sonication (Heat Systems sonicator on power output 4 at 40% duty cycle using a microprobe tip). The disrupted lysate was mixed with 1 ml of a 90% sucrose solution in MBS and transferred to a polyallomer centrifuge tube. A discontinuous gradient of 35%, 25%, 15%, and 5% sucrose in MBS containing 250 mM sodium carbonate was layered on top of the sample. Centrifugation was carried out at 164,000×g for 16-20 h. After centrifugation, fractions (~ 0.4 ml) were removed from the top of the tube and neutralized with 1 N hydrochloric acid. Protein was determined using the BCA protein assay (Pierce) for each fraction, and the samples were stored at -80°C for further analysis.

**Immunoblotting** – Samples from the detergent-free, non-alkaline and alkaline methods of caveolae isolation were analyzed by dot blot. Samples were diluted in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20 (TBST) containing 0.5 mM
dithiothreitol and spotted on nitrocellulose. The membrane was washed and blocked in 5% non-fat dry milk in TBST (blocking buffer), rinsed in TBST, and then incubated (60 min) with the primary antibody (i.e. anti-caveolin, anti-eNOS, anti-argininosuccinate synthase, anti-argininosuccinate lyase or anti-GM130) prepared in blocking buffer. Following extensive washing with TBST, membranes were incubated (60 min) with the secondary antibody (anti-rabbit IgG HRP conjugated antibody) in blocking buffer. After washing with TBST, the membrane was incubated in ECL reagent (Amersham Pharmacia Biotech Inc.) according to the manufacturer’s instructions and then exposed to film.

Polyacrylamide Gel Electrophoresis and Western Blotting – Samples from the detergent-free alkaline method of caveolae isolation were precipitated with trichloroacetic acid and resuspended in a sample buffer containing sodium dodecyl sulfate (SDS), β-mercaptoethanol and tracking dye. Equal amounts of protein (except where noted) were loaded in each lane of a precast 12% Tris-HCl mini-gel (Bio-Rad Laboratories). The gel was run according to the manufacturer’s instructions and then the proteins were transferred to Immobilon-P membranes (Millipore Corporation) using a wet transfer method (Bio-Rad Laboratories). The membranes were incubated with antibodies against caveolin-1, endothelial nitric oxide synthase, argininosuccinate synthase, and GM130 following the protocol outlined in the immunoblotting section above. Also, a peptide neutralization procedure was performed with the anti-AS antibody and 5x AS peptide (by weight) to show specificity of the antibody.

Statistical Analyses – Experimental values are expressed as mean ± SE. At least three independent experiments were performed for quantitative analyses of nitric oxide production and amino acid determinations.
Results

The Effect of Exogenous Arginine or Citrulline on Nitric Oxide Production in Bradykinin-Stimulated Endothelial Cells Grown in an Arginine Deficient Medium –

Cultured endothelial cells are typically maintained in media containing 0.5-1.1 mM L-arginine. In comparison, normal plasma arginine concentrations are approximately 0.1-0.2 mM (Castillo et al. 1993; Wu & Morris 1998a). Such high concentrations of extracellular L-arginine could affect L-arginine utilization by endothelial cells, particularly in relation to nitric oxide production. Therefore, we examined the relationship between intracellular and extracellular L-arginine concentrations on nitric oxide production in cultured endothelial cells (BAEC). Thirty minutes prior to stimulation with bradykinin (10 µM), the media was changed to a synthetic DMEM media containing no serum and the indicated levels of either L-arginine or L-citrulline, respectively. As shown in Fig. 1, incubation of BAEC with various concentrations of L-arginine resulted in a significant increase (~5- to 10-fold) in nitric oxide production for values from 125 to 500 µM. When the same experiment was carried out with citrulline, in place of arginine, a similar increase in nitric oxide production was observed. The measured nitric oxide activity was inhibited by the addition of the eNOS inhibitor, L-NAME (data not shown, Appendix D).

The Effect of Exogenous Citrulline or Arginine on Intracellular Arginine and Citrulline Levels in Bradykinin-Stimulated Endothelial Cells – To determine whether changes in intracellular L-arginine content contributed to the changes in nitric oxide production, intracellular arginine and citrulline levels were determined using HPLC analysis. As shown in Fig. 2A, incubation of BAEC in a synthetic media with 0 to 1000 µM L-arginine resulted in a dose-dependent increase in intracellular arginine. However, the intracellular concentrations of citrulline were not influenced by changes in the level of extracellular or intracellular arginine. Similarly, when extracellular citrulline was varied, intracellular arginine levels were not significantly affected in spite of the fact that enhanced nitric oxide release was nearly identical to that caused by varying extracellular arginine (Fig. 2B). This result suggested that citrulline was efficiently converted to arginine maintaining the arginine required to support nitric oxide production. The fact
that exogenous arginine or citrulline effectively sustained nitric oxide production in
BAEC also suggested that the arginine necessary to support nitric oxide production could
be efficiently maintained by extracellular arginine or citrulline even though “bulk”
intracellular arginine levels appeared to be sufficient to “saturate” eNOS. Most
importantly, though, citrulline efficiently mimicked the arginine effect on enhanced nitric
oxide production.

The Effect of Exogenous Citrulline or Arginine on Nitric Oxide Production in 
Bradykinin- Stimulated Endothelial Cells Grown in Complete Medium – The
proficiency of exogenous arginine or citrulline to support nitric oxide production in
medium lacking arginine was compared with their capacity to support nitric oxide
production in medium where arginine was not limiting. In medium lacking arginine (Fig.
3A), both exogenous arginine (500 µM) and citrulline (500 µM) enhanced nitric oxide
production. However, under conditions where the same medium now contained 443 µM
L-arginine (comparable to the levels normally found in DMEM medium, Fig. 3B),
citrulline enhanced nitric oxide production in spite of the fact that both endogenous and
exogenous arginine levels were saturating and therefore sufficient to support nitric oxide
production. Moreover, the demonstration that additional arginine (500 µM) did not
further enhance nitric oxide production (compare Figs. 3A and 3B) in the arginine
supplemented medium supported the conclusion that the uptake of exogenous arginine
was saturating under these conditions. Note that the control sample of Fig. 3B is
equivalent to the ‘Arg’ sample of Fig. 3A. All measurable nitric oxide production was
inhibited by the eNOS inhibitor, L-NAME.

Interestingly, the intracellular arginine and citrulline levels for any given
concentration of exogenous addition (data not shown) were found to be identical to those
(corresponding levels shown in Fig. 2. Again, intracellular citrulline levels remained
unaffected by the increased intracellular arginine that resulted from the addition of
exogenous arginine, and most significantly, the addition of citrulline (500 µM) did not
affect intracellular arginine levels despite the observed enhanced production of nitric
oxide. The fact that there was a proportionate increase in nitric oxide production with the
addition of citrulline by stimulated endothelial cells saturated in arginine also suggested
that citrulline was limiting and that the generation of arginine from citrulline provided the
substrate, arginine, for nitric oxide production.

The Co-Fractionation of Nitric Oxide Synthase, Argininosuccinate Synthase
and Argininosuccinate Lyase with Plasmalemmal Caveolae – Definition of a unique
pool of arginine may occur through the functional association of enzymes involved in the
generation of arginine from citrulline. To address this possibility, a caveolar fraction was
generated by successive gradient fractionations using a detergent-free, non-alkaline
procedure for purifying caveolae membranes (Smart et al. 1995). As shown in Fig. 4,
where membrane and caveolae fractions were followed by immunoblotting, both eNOS
as well as argininosuccinate synthase and lyase were identified in the caveolar fraction.
Although all immuno-crossreacting material for argininosuccinate synthase and lyase co-
fractionated with the membrane fraction in the initial gradient, the association of
argininosuccinate synthase and lyase in the final caveolar fraction was not quantitative.
This result was not unanticipated considering argininosuccinate synthase and
argininosuccinate lyase are not integral membrane proteins, nor do they possess
myristoylation or palmitoylation that would secure their association with eNOS in the
caveolar plasmalemmal fraction. Probable protein-protein interactions that are likely
involved in their plasmalemmal localization with nitric oxide production may not
quantitatively withstand the rigors of all the gradient fractionation steps. There was no
Golgi matrix protein crossreacting material found in the second gradient step indicating
that this fraction was not contaminated by Golgi membrane (data not shown).

To ensure that the co-localization observed was not simply intrinsic to the
caveolar purification protocol, a different caveolar protocol with very different
fractionation conditions was used. In this procedure, after cell lysis and homogenization,
a single alkaline sucrose step-gradient separates caveolae from the other membranes and
cellular material (Song et al. 1996). As shown in Fig. 5, both argininosuccinate synthase
and argininosuccinate lyase were again shown to co-
fractionate with caveolae, even
under relatively extreme alkaline conditions. Golgi membrane, based on an anti-Golgi
matrix protein antibody, was detected only in fractions 5 through 7 of the alkaline
gradient (data not shown) corresponding to approximately 22-26% sucrose where the
Golgi membrane was expected to fractionate (Vleurick et al. 1999). Results, showing that cellular argininosuccinate synthase and argininosuccinate lyase remain associated with a highly purified caveolar plasmalemmal fraction, are consistent with the proposal that the regeneration of arginine from citrulline defines a unique caveolar pool of arginine utilized by vascular endothelial cells for nitric oxide signaling.

The identity of argininosuccinate synthase, eNOS and caveolin-1 was confirmed by Western blotting of fractions separated by the alkaline caveolae membrane isolation procedure. As shown in Fig. 6, the Western blot data shows co-fractionation of caveolin-1, eNOS and argininosuccinate synthase proteins. The amount of argininosuccinate lyase present in the caveolae membrane fraction after SDS-PAGE and transfer was below the primary antibody limit of detection by Western blotting. A marker protein for the Golgi, the GM130 protein, was detected in fractions 5 through 10 of the alkaline gradient (data not shown) (Vleurick et al. 1999). The protein profile shows that the bulk of the protein remains in the more dense gradient fractions (fractions 6-10).
Discussion

We have shown that exogenous citrulline effectively stimulated nitric oxide production, and that it was sufficient to affect maximal endothelial nitric oxide production in spite of apparent saturating levels of both endogenous and exogenous arginine. Exogenous citrulline did not affect intracellular arginine levels, indicating that there was no direct correlation between bulk intracellular arginine levels and nitric oxide production. These findings were taken to support the proposal that the regeneration of arginine from citrulline is necessary and sufficient in providing the substrate, arginine, for nitric oxide production. By defining a unique source of arginine for nitric oxide production (Fig. 7), the endothelial cell spares the bulk of cellular arginine for other metabolic pathways. In support of this proposal are reports demonstrating that the inhibition of argininosuccinate synthase in cultured endothelial cells blocked production of arginine, and consequently blocked nitric oxide production in spite of intracellular arginine levels sufficient to support nitric oxide production (Hecker et al. 1990a; Hecker et al. 1990b; Sessa et al. 1990b).

If regeneration of arginine from citrulline defines the source of arginine for nitric oxide production, this would suggest that these two processes are functionally associated. Since endothelial nitric oxide synthase is targeted by acylation to caveolae, where it interacts with caveolin-1, we asked the question as to whether the arginine regenerating enzymes, argininosuccinate synthase and argininosuccinate lyase, co-localize with this same subcellular fraction. Two different fractionation protocols for the purification of caveolae were used to demonstrate co-fractionation (Smart et al. 1995; Song et al. 1996). Each protocol generated a caveolar membrane fraction that was highly enriched in caveolin-1, nitric oxide synthase, argininosuccinate synthase and argininosuccinate lyase. These results, therefore, were taken to support the proposal that the endothelial cell can effectively distinguish the bulk of intracellular arginine from the arginine needed for nitric oxide production through the functional co-localization of arginine regeneration enzymes and nitric oxide synthase with plasmalemmal caveolae. Although not demonstrated in these results, a possible consequence of this functional association would be the channeling of intermediates through these regeneration enzymes, similar to that
observed for urea cycle intermediates, where essentially no metabolite between citrulline to arginine would be found in an intracellular pool of the endothelial cell.

The only known metabolic fate of citrulline is as a substrate for argininosuccinate synthase. Conjugation of citrulline with aspartate, catalyzed by argininosuccinate synthase, provides the amino nitrogen that ultimately is released as nitric oxide from arginine. Arginine formed by argininosuccinate lyase from argininosuccinate would be channeled to nitric oxide synthase; whereas, fumarate could be recycled to oxaloacetate. Oxaloacetate, in turn, can accept another amino group to reform aspartate (Fig. 7). Thus, in endothelial cells, the funneling of amino groups from other amino acids into either aspartate (or glutamate) provides the nitrogen for nitric oxide production. In this way, the backbone of citrulline/arginine is conserved and the expendable nitrogen for nitric oxide derives from the transamination of amino groups from other amino acids into aspartate.
Acknowledgments

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Figure 1. Effect of L-arginine and L-citrulline on bradykinin stimulated nitric oxide production in DMEM minus arginine. Endothelial cells were grown to confluence (6-well cluster dishes; 9.6 cm²/well) in complete DMEM as described in Experimental Procedures. As indicated, concentrations of L-arginine or L-citrulline were added to their respective wells (in duplicate) in a total of 2 ml of synthetic DMEM (minus arginine) containing 2.5 mM CaCl₂. After 30 min, 100 µl of media was removed for nitrite determination, and, where indicated, the cells were stimulated by the addition of bradykinin (final concentration of 10 µM). Incubation was continued for an additional 60 min at which time samples (100 µl) of media were taken and assayed for nitric oxide production (nitrite determination). Conditions are indicated in each panel. Error bars represent the standard error of the mean.
Figure 2. Effect of exogenous arginine or citrulline on intracellular concentrations of arginine and citrulline. Endothelial cells were grown in DMEM as described in Experimental Procedures. The indicated concentration of L-arginine or L-citrulline was added in a total of 2 ml of synthetic DMEM (minus arginine) containing 2.5 mM CaCl$_2$. After 30 min, the cells, where indicated, were stimulated by the addition of bradykinin (final concentration of 10 μM). Incubation was continued for an additional 60 min. Cells were lysed in 90% methanol, and the soluble fraction derivatized by PITC. PITC derivatized samples were fractionated on a 5 µm C-18 reverse phase column (4.6 mm x 250 mm) (Supelco) as described in Experimental Procedures. Integrated values for arginine and citrulline peaks were normalized against an internal standard (threonine) to assess changes. (A) Levels of intracellular arginine and citrulline affected by the addition of arginine. (B) Levels of intracellular arginine and citrulline affected by the addition of citrulline. Arginine levels: plus bradykinin (filled circles), minus bradykinin (open circles). Citrulline levels: plus bradykinin (filled triangles), minus bradykinin (open triangles).
Figure 3. Effect of arginine and citrulline on bradykinin stimulated nitric oxide production in the absence (Fig. 3A) and presence (Fig. 3B) of arginine in the medium. Endothelial cells were grown as described in Experimental Procedures. L-arginine (‘Arg’, 500 µM) or L-citrulline (‘Cit’, 500 µM) was added in a total of 2 ml of synthetic DMEM (plus or minus 443 µM L-arginine) containing 2.5 mM CaCl₂. After 30 min, 100 µl of media was removed for nitrite determination, and the cells were stimulated, where indicated, by the addition of bradykinin (‘Bk’, 10 µM). Incubation was continued for another 60 min at which time samples (100 µl) of media were taken and assayed for nitric oxide production (nitrite determination). The amount of nitrite found in the sample at the 30 min equilibration time point was subtracted from the amount of nitrite found at the 90 min time point. This value was converted to pmol of nitrite formed in 60 min per number of cells in each plate well. ‘C’ represents control in synthetic DMEM. Error bars represent the standard error of the mean.
Figure 4. Detergent-free, non-alkaline, co-fractionation of plasmalemmal caveolae, nitric oxide synthase, argininosuccinate synthase and argininosuccinate lyase. Fractions from the last 5% OptiPrep centrifugation step (see Experimental Procedures) were collected from the top of the tube and analyzed by immunoblotting using the appropriate primary antibody, and visualized by enhanced chemiluminescence. Boxed areas represent the plasmalemmal caveolar fractions. Fraction numbers are in ascending order from the top to the bottom of the tube.
Figure 5. Alkaline sucrose co-fractionation of plasmalemmal caveolae, nitric oxide synthase, argininosuccinate synthase and argininosuccinate lyase. After centrifugation, fractions (~ 0.4 ml) were collected from the top of the gradient and analyzed by immunoblotting using the appropriate primary antibody, and visualized by enhanced chemiluminescence. Boxed areas represent the plasmalemmal caveolar fractions. Fraction numbers are in ascending order from the top to the bottom of the tube.
Figure 6. Western blotting with caveolin-1, endothelial nitric oxide synthase, and argininosuccinate synthase. Gradient fractions from an alkaline sucrose caveolae membrane preparation were electrophoresed and then analyzed by Western blotting using the appropriate primary antibody, and visualized by enhanced chemiluminescence. Boxed areas represent the plasmalemmal caveolar fractions. Fraction numbers are in ascending order from the top to the bottom of the tube. In the Western blots showing caveolin-1 and eNOS co-fractionation, 3 µg of total protein from Fractions 1-2 and 10 µg of total protein from Fractions 3-11 were loaded on the gel. In the Western blot illustrating argininosuccinate synthase co-fractionation, the following total protein amounts were loaded on the gel: Fraction 1: 2 µg, Fraction 2: 8 µg and Fractions 3-11: 21 µg. Prestained protein marker apparent molecular weights (kDa) are labeled at the left of each blot. Also shown at the bottom of the figure is a graph of the gradient fraction protein profile.
Figure 7. Arginine regeneration for nitric oxide production in vascular endothelial cells. The integration of pathways for the synthesis of nitric oxide, regeneration of arginine from citrulline, and the recycling of fumarate to aspartate are illustrated in the diagram.
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Paper II:
Protein-Protein Interactions in the Citrulline-Nitric Oxide Cycle

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Abstract

Nitric oxide (NO), a key vasorelaxant, is formed by sequential reactions where arginine is converted to NO and citrulline by endothelial nitric oxide synthase (eNOS). Citrulline is regenerated to arginine by argininosuccinate synthase (AS) followed by argininosuccinate lyase (AL). This series of reactions constitutes the citrulline-NO cycle, which plays an essential role in endothelial NO production. The enzymes of the citrulline-NO cycle are associated with caveolae but there is no evidence for direct protein interactions between these components. AS and AL were cloned and expressed in E. coli with two different N-terminal fusion tags, a glutathione S-transferase (GST) or a hexahistidine, T7 (6xHis-T7) tag. Bovine aortic endothelial cells (BAEC) were either untreated or stimulated with a calcium agonist to increase NO production. Endothelial cell lysates were prepared and used to test for protein-protein interactions using in vitro interaction assays with bacterially expressed fusion proteins. AS and AL with 6xHis-T7 tags pulled down eNOS and caveolin-1 from a BAEC lysate. These results provide, for the first time, evidence for a physical association between AS, AL, eNOS and caveolin-1, consistent with the important functional role of the citrulline-NO cycle in endothelial NO production.

Keywords: nitric oxide, argininosuccinate synthase, argininosuccinate lyase, citrulline, arginine
Introduction

Nitric oxide (NO), is an essential signaling component for a broad array of physiological processes important for immune, neurological and cardiovascular functions. It is produced by the conversion of arginine to NO and citrulline in a reaction catalyzed by nitric oxide synthase (NOS). The endothelial (eNOS) and neuronal (nNOS) forms of NOS are largely constitutive and are activated by association with Ca-calmodulin in response to transient increases in intracellular Ca\(^{2+}\) levels, while the inducible form is regulated primarily at the transcriptional level. NO produced by the endothelium plays a key role in vasorelaxation. Because of the important role of endothelial-derived NO, eNOS is regulated at several different levels including translational, post-translational, protein-protein interactions, cofactor availability and substrate (arginine) availability. The product, citrulline, is regenerated to arginine by the sequential action of argininosuccinate synthase (AS) and argininosuccinate lyase (AL), two enzymes which have received a great amount of study for their role in the urea cycle. In 1990, Vane and colleagues, and subsequently others, observed that in cultured endothelial cells in the presence of saturating amounts of arginine, the addition of exogenous arginine caused endothelial cells to produce more NO (Hecker et al. 1990; Wu et al. 1998; Wu et al. 1993). This is surprising given that the K\(_m\) of eNOS for arginine is ~3 µM and intracellular endothelial arginine levels average between 100-800 µM. This phenomenon is termed the “arginine paradox”.

Van Geldre et al. showed that in muscle layer nerve fibers and neurons in the myenteric plexus of the rat gastric fundus, AS and AL co-localized with nNOS, another calcium-dependent NOS. They also showed in smooth muscle strips that administration of arginine completely, and citrulline partially, prevented L-NAME (NOS inhibitor) inhibition of muscle relaxation (Van Geldre et al. 2002). Mohacsi et al. demonstrated that arginine availability was not limiting for recombinant eNOS activity (Mohacsi et al. 1999). Adenovirus mediated eNOS transfer into rabbit aorta was performed and the researchers noted improved vascular relaxation with or without arginine administration. Simon et al. demonstrated that there are different intracellular compartments of arginine depending on cell type and eventual fate (Simon et al. 2003). McDonald et al.
demonstrated using confocal microscopy in porcine aortic endothelial cells that caveolin-1, the structural protein of caveolae, colocalized with the cationic amino acid transporter 1, system y+ (CAT1) and that eNOS and CAT1 colocalized to caveolae. They concluded that the intracellular arginine was inaccessible to eNOS and that eNOS was colocalized with the arginine transporter, CAT1, at the plasma membrane surface to allow for NO production from arginine transported into the cell (McDonald et al. 1997). More recently, Li et al. established that eNOS and CAT1 directly interact, but that this interaction increases NO production in an arginine transport independent manner (Li et al. 2005). Hellermann et al. examined the effects of sodium orthovanadate (vanadate), a protein tyrosine phosphatase. They demonstrated that stimulation of NO production by bradykinin and sodium orthovanadate, greatly increased NO production over the basal state which they attributed to changes in eNOS protein-protein interactions (Hellermann et al. 2000). Venema et al. also showed an interaction through heat shock protein 90 (hsp90) between eNOS and soluble guanylate cyclase which is activated by NO binding to the heme within the enzyme (Venema et al. 2003). Jiang et al. showed that in COS-7 cells transfected with eNOS and carboxyl terminus of Hsp70-interacting protein (CHIP), a co-chaperone protein of hsp90, CHIP was incorporated into the eNOS-hsp90 complex and then soluble eNOS was segregated to an insoluble and inactive cellular compartment (Jiang et al. 2003). The eNOS interacting protein, NOSIP, was also shown to associate with eNOS causing it to translocate from the caveolae to other subcellular fractions (Dedio et al. 2001). Zimmermann et al. demonstrated that overexpression of eNOS traffic inducer, NOSTRIN, also caused a translocation of eNOS away from the plasma membrane resulting in reduced NO production in Chinese hamster ovary cells stably expressing eNOS (Zimmermann et al. 2002). Our laboratory demonstrated a co-fractionation of eNOS, AS and AL with caveolin-1 from endothelial cell caveolar membranes (Flam et al. 2001).

To complement our results and the results of others outlined above, we designed a series of *in vitro* interaction assays to assess direct protein-protein interactions and those associations that may occur as the result of post-translational modifications. We prepared AS and AL bait proteins with either 6xHis or GST N-terminal fusion tags. *In vitro*
translated AS and endothelial cell lysates were used as prey proteins. We demonstrated a
direct interaction between AS and AL and associations between AS and eNOS; AL and
eNOS; AS and caveolin-1; and AL and caveolin-1. These results further support our
hypothesis of a functional association between enzymes of the citrulline-NO cycle. These
functional associations provide strong evidence for a physical association between AS,
AL, eNOS and caveolin-1, consistent with the important functional role of the citrulline-
NO cycle in endothelial NO production.
Cloning of bovine argininosuccinate synthase (bAS) into plasmid pGEX-5X-1 and pET-28b(+) – bAS-pcDNA3.1-V5HisB, a plasmid encoding bAS with carboxy-terminal V5 and hexahistidine fusion tags, was previously prepared. The coding portion of bAS was amplified from bAS_pcDNA3.1_V5HisB using primers bASL_BamHI_pGEX (5’ – GTA CCG AGC TCG GAT CCG CCC CTG CTC CGC CGA CTG – 3’) and ASR1248EcoRI (5’ – TGC AGA ATT CTT TTT GGC GGT GAC CTT GTT CTG – 3’) synthesized by IDT. PCR was accomplished using Pfu Turbo DNA polymerase (Stratagene) and 30 cycles with a 2 minute extension time. The Qiaquick PCR cleanup kit (Qiagen) was used to remove excess nucleotides and the bAS PCR product, pGEX-5X-1 (GE Healthcare) and pET-28b(+) (Novagen) were doubled digested with BamHI and EcoRI. The bAS PCR product (1419 bp) was ligated into the pGEX-5X-1 and pET-28b(+) plasmids using the LigaFast Rapid DNA ligation system (Promega) and then transformed into E. coli DH5α (Invitrogen). Colonies were analyzed and a positive clone from each group, pGEX-5X-1-bAS or pET-28b(+)bAS, was sequenced (Retrogen).

Cloning of human argininosuccinate lyase (hAL) into plasmid pGEX-5X-1 and pET-28c(+) – The plasmids pAL3b_pUC8 (ATCC 57497, accession number M14218), pGEX-5X-1 and pET-28c(+) were digested with BamHI. The digested plasmids, pGEX-5X-1 and pET-28c(+), were treated with calf alkaline phosphatase (Promega) to prevent plasmid religation. The hAL insert (1628 bp) was ligated into the pGEX-5X-1 and pET-28c(+) plasmids using the LigaFast Rapid DNA ligation system and then transformed into DH5α. Colonies were analyzed and a positive clone, pGEX-5X-1-hAL or pET-28c(+)hAL, from each group was sequenced.

Expression of fusion proteins – All the previously prepared plasmids in either pGEX-5X or pET-28 were transformed into E. coli Rosetta 2 or Rosetta 2(DE3), respectively, competent cells (Novagen). A 10 ml culture of Rosetta 2 was grown in P-0.5G minimal medium according to the method of Studier (Studier 2005) in a 125 ml flask at 225 rpm and 37°C in an incubator shaker (New Brunswick Scientific). 5 ml of this culture was added to ZYP-5052 medium, an auto-induction medium containing 1% tryptone, 0.5% yeast, 0.5% glycerol, 0.05% glucose, 0.2% α-lactose and trace metals
(FeCl₃, CaCl₂, MnCl₂, ZnSO₄, CoCl₂, CuCl₂, NiCl₂, Na₂MoO₄, Na₂SeO₃ and H₃BO₃). The ZYP-5052 medium also contained 34 µg/ml chloramphenicol and either 100 µg/ml carbenicillin for pGEX plasmids or 30 µg/ml kanamycin for pET-28 plasmids. 500 ml of each culture was grown in a 2 liter flask at 200 rpm and 22°C in an incubator shaker. The pGEX-5X-1, pGEX-5X-1-hAL, pGEX-5X-1-bAS, pET28c(+)-hAL and pET28b(+)-bAS cultures in ZYP-5052 medium were grown for 48 hours. The cells were collected by centrifugation at 6000xg for 20 minutes and stored frozen at -20°C until use.

Purification of GST fusion proteins – Cell pellets were thawed on ice and 15 ml lysis buffer A (50 mM Tris-HCl, pH 7.8; 1 mM EDTA; 10% glycerol (v/v); 150 mM NaCl) containing 1.5 ml of mini EDTA-free protease inhibitor cocktail tablet (Roche) and 200 µg/ml of lysozyme was added to each 500 ml culture pellet. The bacterial suspension was lysed by stirring at 4°C for 20 minutes followed by pulsed sonication. The sonicated bacterial suspension was centrifuged at 25,000xg for 30 minutes at 4°C to pellet insoluble material. For GST, GST-bAS and GST-hAL protein purification, the following steps were performed. Six ml of glutathione-agarose slurry (50% slurry in lysis buffer A, Pierce Biotechnology) was added to the soluble fraction and placed on a Nutator rocker for 1 hour at 4°C. The soluble fraction and glutathione-agarose were centrifuged to pellet the glutathione-agarose beads. After five washes with glutathione wash buffer (50 mM Tris-HCl, pH 7.8; 400 mM NaCl), enough buffer was removed to create a 50% slurry. Fusion proteins bound to agarose beads were stored at 4°C.

Purification of 6xHis-T7 tag fusion proteins – 6xHis bacterial pellets were thawed on ice and resuspended in 15 ml of lysis buffer B (50 mM Tris Base; 300 mM NaCl; 5 mM imidazole, pH 8.0) containing 1.5 ml of mini EDTA-free protease inhibitor cocktail tablet and 200 µg/ml of lysozyme. The bacterial suspension was lysed by stirring at 4°C for 20 minutes followed by pulsed sonication. The sonicated bacterial suspension was centrifuged at 25,000xg for 30 minutes at 4°C to pellet insoluble material. For 6xHis-bAS and 6xHis-hAL protein purification, the following steps were performed. Six ml of nickel-nitriloacetic acid (Ni-NTA) agarose slurry (50% slurry in lysis buffer B, Qiagen) was added to the soluble fraction and placed on a Nutator rocker for 1 hour at 4°C. The soluble fraction and Ni-NTA agarose were centrifuged to pellet the Ni-NTA agarose
beads. After five successive washes each with wash buffer 1 (50 mM Tris Base; 300 mM NaCl; 10 mM imidazole, pH 8.0), wash buffer 2 (50 mM Tris Base; 300 mM NaCl; 20 mM imidazole, pH 8.0) and wash buffer 3 (50 mM Tris Base; 300 mM NaCl; 45 mM imidazole, pH 8.0) the buffer was removed and lysis buffer B was added back to create a 50% slurry. Fusion proteins bound to agarose beads were stored at 4°C.

**In vitro transcription and translation** – bAS-pcDNA3.1-V5HisB was *in vitro* translated using the TNT T7 Coupled Reticulocyte Lysate System (Promega) according to the manufacturer’s protocol.

**Cell culture and reagents** – Bovine aortic endothelial cells (BAEC) in 100 mm dishes were cultured in Dulbecco’s modification of Eagle’s medium (DMEM) containing 10% fetal bovine serum (fbs), 100 IU penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B at 37°C in a 5% CO₂ incubator. DMEM, HBSS, HEPES, and L-glutamine were obtained from Mediatech, Inc. Fetal bovine serum was obtained from HyClone. DMEM without phenol red was obtained from Invitrogen. Bradykinin was obtained from Sigma-Aldrich. Sodium orthovanadate was obtained from Alexis Biochemicals.

**BAEC lysate preparation** – On the day of the experiment, BAEC were rinsed in Hanks’ balanced salt solution without phenol red containing 20 mM HEPES. Phenol red-free DMEM containing 10% fbs and 4 mM L-glutamine was added to each dish. Some cells were treated with 10 µM bradykinin plus 20 µM sodium orthovanadate for 90 minutes. A portion of medium was removed for nitric oxide determinations at the beginning and end of the treatment. Following the treatment, the dishes were placed on ice, medium was removed and the cells were washed with cold 1x Dulbecco’s phosphate buffered saline (Mediatech). Modified RIPA buffer (20 mM Tris-HCl, pH 7.4; 1% Triton X-100; 1% sodium deoxycholate; 0.1% SDS; 1 mM sodium orthovanadate, 50 mM sodium fluoride; 10 mM sodium pyrophosphate; 1x Calbiochem protease inhibitor cocktail III) was added and the cells were scraped off the dish. The cell suspension was removed to a 2 ml microfuge tube and the suspension was disrupted by pulsed sonication for six seconds at 20% duty cycle. Protein content was determined using the BCA Protein Assay (Pierce Biotechnology) and the lysates were stored at -80°C until used.
In vitro interaction assays – In vitro translated proteins as bait – After the bAS and hAL fusion proteins were prepared, 40 µl of GST fusion protein bound to glutathione-agarose was added to 1.5 ml microfuge tubes. 20 µl of in vitro translated protein was added, the final volume was brought to 1 ml using binding buffer and the mixture was then placed on end-over-end rotator for two hours at 4°C. Following binding, the agarose beads were pelleted by centrifugation at 1000xg for 5 minutes at 4°C. Pull-down assay tubes containing GST fusion proteins bound to glutathione-agarose were washed six times with wash buffer A (50 mM HEPES, pH 7.5; 400 mM NaCl; 0.5% CHAPS detergent; 1 mM AEBSF). The final wash was removed and 25 µl of 2x Laemmli sample buffer containing 320 mM dithiothreitol was added to each sample.

In vitro interaction assays – BAEC lysates as bait – After the bAS and hAL fusion proteins were prepared, 20 µl of 6xHis fusion protein bound to Ni-NTA agarose was added to 1.5 ml microfuge tubes. 250 µg of BAEC lysate was added, the final volume was brought to 1 ml using binding buffer (50 mM Tris-HCl, pH 7.4; 20% glycerol; 100 mM NaCl; 20 mM 2-mercaptoethanol; Calbiochem 1x protease inhibitor cocktail III) and the mixture was then placed on end-over-end rotator for two hours at 4°C. Following binding, the agarose beads were pelleted by centrifugation at 1000xg for 5 minutes at 4°C. Pull-down assay tubes containing 6xHis fusion proteins bound to Ni-NTA agarose were washed six times with wash buffer B (50 mM HEPES, pH 7.5; 10 mM imidazole, pH 7.5; 400 mM NaCl; 0.5% CHAPS detergent; 1 mM AEBSF). The final wash was removed and 25 µl of 2x Laemmli sample buffer (Bio-Rad Laboratories) containing 320 mM dithiothreitol was added to each sample.

Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting – Samples in reducing Laemmli sample buffer were heated for 5 minutes at 95°C, cooled to room temperature and loaded on a 4-15 % Tris-HCl gradient gel (Bio-Rad Laboratories). The proteins were separated using a Mini-Protean II (Bio-Rad Laboratories) following the manufacturer’s instructions. Following SDS-PAGE, the proteins were transferred to Immobilon-P polyvinylidene fluoride (PVDF) membranes (Millipore) using a Mini-Protean transfer system (Bio-Rad Laboratories) and following the manufacturer’s instructions. After the transfer, the membrane was removed and
blocked in 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 hour at room temperature. The SDS-PAGE gel was soaked in water to remove SDS and to dissociate the filter paper from the gel. The gel was then stained with GelCode Blue to determine protein loading. Primary antibody prepared in 5% non-fat dry milk/TBST was added to the membrane and incubated overnight at 4°C. The primary antibodies that were used included anti-V5, mouse monoclonal (Invitrogen; 1:5000); anti-GST, mouse monoclonal (Santa Cruz Biotechnology; 1:4000); anti-His-tag, mouse monoclonal (Novagen; 1:5000); anti-AS, mouse monoclonal, (BD Transduction Laboratories; 1:2500); anti-caveolin1 monoclonal, (BD Transduction Labs; 1:1000) and anti-eNOS, mouse monoclonal (BD Transduction Labs; 1:2500). The membrane was then rinsed with TBST several times. A secondary antibody prepared in 5% non-fat dry milk/TBST was added to the membrane and incubated for 1 hour at room temperature. The secondary antibody used was peroxidase-conjugated affinipure goat anti-mouse IgG, (Jackson ImmunoResearch Laboratories, Inc). The membrane was again washed with TBST and proteins were detected using ECL (GE Healthcare).

**SDS-PAGE and staining** – 30 µl of 2x reducing Laemmli sample buffer was added to 15 µl of fusion protein bound to agarose beads. The samples were heated for 5 minutes at 95°C, cooled to room temperature and loaded on a 4-15 % Tris-HCl gradient gel. The proteins were separated using a Mini-Protean II following the manufacturer’s instructions. Following SDS-PAGE, the gel was water washed and stained with GelCode Blue to visualize total protein loading. The gel was fixed in 2% glycerol, 10% methanol and dried on Whatman 3MM filter paper. The gel was scanned and band densitometry was performed using ImageQuant 5.2 (GE Healthcare).

**Statistics** – The results shown are representative immunoblots from experiments repeated at least two times.
Results

Expression of bAS and hAL fusion proteins – Fusion proteins encoded by the pET-28 vectors contained either an N-terminal 6xHis-thrombin-T7 tag plus an optional C-terminal 6xHis (6xHis). The plasmid conferred resistance to 30 µg/ml kanamycin and protein expression was controlled by a T7 promoter. Fusion proteins encoded by the pGEX-5X vectors contained an N-terminal glutathione-S-transferase-factor Xa cleavage tag (GST). The plasmid conferred resistance to 100 µg/ml ampicillin and protein expression was controlled by a tac promoter. Table 1 indicates plasmid designation, fusion protein name and theoretical size (Gasteiger et al. 2005). Using the Gap program the percent similarity between bAS and hAS was 97% and between bAL and hAL was 96% (Seqweb 3.1, Accelrys, Inc.)

After the fusion protein plasmids were sequenced, the pET-28 plasmids were transformed into Rosetta 2(DE3) and the pGEX-5X plasmids were transformed into Rosetta 2 competent bacteria for protein expression. The Rosetta 2 strain is derived from BL21 and contains the pRARE plasmid conferring resistance to chloramphenicol. This pRARE plasmid contains tRNA genes that decode for seven rare codons, improving full length eukaryotic protein expression. Originally some of the plasmids were transformed into BL21(DE3)pLysS for protein expression, but greater protein yields were obtained with the Rosetta 2 strains. Also, the use of 2 liter flasks for the 500 ml cultures improved aeration, growth and protein yield (Studier 2005).

After extensive washes and batch purification, fusion proteins bound to agarose beads were mixed with reducing sample buffer and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred from the gel to PVDF membranes and fusion proteins were detected by immunoblotting (Figures 1B-C). The polyacrylamide gel was stained with GelCode Blue after transfer to visualize those proteins remaining in the gel (Figure 1A).

In vitro interaction assay with in vitro translated protein as prey – An in vitro interaction assay was performed using GST fusion protein as bait and in vitro translated bAS-V5-6xHis protein as prey as described in the methods section. Proteins were transferred from the gel to PVDF membranes and the in vitro translated bAS-V5-6xHis
was detected by immunoblotting with antibodies against V5 (Figure 2A) and AS (Figure 2B). After transfer, the polyacrylamide gel was stained with GelCode Blue to visualize proteins remaining in the gel (Figure 2C). These data show a direct interaction between \textit{in vitro} translated AS and bacterially expressed AL. This is not surprising since both AS and AL are components of the citrulline-NO cycle and both exist as homotetrameric proteins (O'Brien 1979; O'Brien et al. 1981).

\textit{In vitro interaction assay with endothelial cell lysate proteins as prey} – An \textit{in vitro} interaction assay was performed using 6xHis fusion protein as bait and endothelial cell lysate as prey as described in the methods section. Proteins were transferred from the gel to PVDF membranes and eNOS was detected by immunoblotting with an antibody against eNOS (Figure 3). Caveolin-1 was detected by immunoblotting with an antibody against caveolin-1 (Figure 4). There was no apparent difference in the amount of eNOS or caveolin-1 from either basal or stimulated endothelial cell lysates that associated with either 6xHis-hAL or 6xHis-bAS. There also was no apparent difference in the amount of eNOS that associated with 6xHis-hAL or 6xHis-bAS from endothelial cells transfected with a plasmid for overexpression of bAS-V5-6xHis (data not shown). However, there were some interactions noticed in the negative control lanes 5-6 (Figure 4A). It was reported by Chatenay-Rivauday et al. that caveolin-1 alpha interacts with the microsomal form of NADH cytochrome B5 reductase which was bound to the Ni-NTA resin and used as a negative control protein (Chatenay-Rivauday et al. 2004). Nevertheless, the signal generated in lanes 5-6 (Figure 4A) is much lower than that in lanes 1-4 (Figure 4A). These data show an association between eNOS and caveolin-1 from endothelial cell lysates with bacterially expressed AS and AL.
Discussion

The *in vitro* interaction assays described provide additional and compelling evidence for protein-protein interactions between enzymes involved in the citrulline-nitric oxide (NO) cycle. The co-localization of argininosuccinate synthase (AS) and argininosuccinate lyase (AL) with neuronal nitric oxide synthase (nNOS) in muscle layer nerve fibers and neurons of the myenteric plexus of the rat gastric fundus (Van Geldre et al. 2002), the immunoprecipitation of tyrosine-phosphorylated eNOS associated proteins upon stimulation (Hellermann et al. 2000) and the interaction between eNOS, heat shock protein 90 (hsp90) and guanylate cyclase (Venema et al. 2003) all support the hypothesis of a protein complex for efficient NO production. Ju et al. also showed a direct interaction between eNOS and caveolin-1 using GST-caveolin-1 as bait and insect cell expressed eNOS as prey protein (Ju et al. 1997). Using a yeast two-hybrid system they were able to map the domains involved in the interaction between the two proteins. Our lab showed a co-fractionation from endothelial cell caveolae, where many signaling molecules are found, of eNOS, AS and AL (Flam et al. 2001).

Proteins that are in close physical association with one another tend to act more efficiently in channeling substrates from one enzyme to another (Cheung et al. 1989). Also, our laboratory recently showed that endothelial NO production is tightly coupled to the citrulline-NO cycle (Paper III). The unique arginine source in endothelial cells, reserved for NO synthesis, does not rely on the bulk intracellular arginine substrate for NO generation. Instead, this unique arginine source is converted by eNOS to NO and citrulline. Citrulline is recycled to arginine by subsequent reactions catalyzed by AS and AL, respectively. When endothelial cells are stimulated with calcium agonists to produce additional NO, the citrulline-NO cycle ensures there is a sufficient arginine supply for endothelial NO production. The ability of the enzymes of the citrulline-NO cycle to efficiently move citrulline through AS and AL without loss of citrulline or argininosuccinate to diffusion becomes critical for arginine regeneration. Most probably the interactions between these enzymes occur dynamically, depending on the state of the endothelial cell and its ability to produce NO (Slon-Usakiewicz et al. 2004).
Through the use of bacterially expressed fusion proteins, we demonstrated a direct interaction between AS and AL and associations between AS and eNOS, AL and eNOS, AS and caveolin-1 and AL and caveolin-1. Further investigations will explore interactions and associations between accessory proteins like hsp90, carboxyl terminus of Hsp70-interacting protein (CHIP), eNOS interacting protein (NOSIP), eNOS traffic inducer (NOSTRIN) or the bradykinin receptor (Dedio et al. 2001; Garcia-Cardena et al. 1997; Jiang et al. 2003; Ju et al. 1998; Ju et al. 1997; Zimmermann et al. 2002). Also, interactions between exact protein domains will be examined using the *in vitro* interaction assays. The knowledge gained from these studies will improve our understanding of how protein-protein interactions in normal and disease states can affect substrate availability, ultimately leading to sufficient or insufficient endothelial NO production.
Acknowledgements

The authors wish to thank Naziya Samreen, an undergraduate student in the Honors College at the University of South Florida for her assistance with cloning and fusion protein purification. This work was supported by American Heart Association Florida Affiliate Grants 9950864V and 0455228B and the University of South Florida Foundation -- Mary and Walter Traskiewicz Memorial Fund.
Table 1. Fusion protein plasmids and protein molecular weight

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Figure 1. Purification of 6xHis and GST fusion proteins. Bacterially expressed 6xHis-hAL and 6xHis-bAS were purified using Ni-NTA agarose as described in the methods. Bacterially expressed rat cytochrome B5 reductase (rCyB5R)-4xHis used as a negative control for the in vitro interaction assays was a gift (G. Roma, University of South Florida). Bacterially expressed GST-hAL, GST-bAS and GST were purified using glutathione agarose as described in the methods. Precision Plus prestained standards (Bio-Rad Laboratories) were used to estimate molecular weight. (A) Proteins were separated by SDS-PAGE under reducing conditions on a 4-15% Tris-HCl gel and following transfer, the gel was stained with Gelcode Blue to visualize total protein loading. (B) An immunoblot of 6xHis fusion proteins following detection with anti-His-Tag monoclonal antibody (Novagen; 1:5000), HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Labs; 1:100,000) and ECL reagent is shown. (C) An immunoblot of GST fusion proteins following detection with anti-GST monoclonal antibody (Santa Cruz Biotechnology; 1:2000), HRP-conjugated goat anti-mouse IgG (1:100,000) and ECL reagent is shown.

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Antibody: anti-His-Tag

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Figure 2. Interactions between *in vitro* translated AS-V5-6xHis and bacterially expressed GST-AL or GST-AS. Bacterially expressed GST-hAL, GST-bAS or GST bound to glutathione-agarose was used as the bait protein. *In vitro* translated bAS-V5-6xHis was used as the prey protein. Precision Plus prestained standards (Bio-Rad Laboratories) were used as approximate molecular weight markers. Proteins were separated by SDS-PAGE under reducing conditions on 4-15% Tris-HCl gels. (A) An immunoblot of bAS-V5-6xHis detected with anti-V5 monoclonal antibody (Invitrogen; 1:5000), HRP-conjugated goat anti-mouse IgG (1:100,000) and ECL reagent is shown. Lane 1 contains 0.1% AS-V5-6xHis input protein. Lane 4 contains the negative control. (B) An immunoblot of bAS-V5-6xHis detected with anti-AS monoclonal antibody (BD Transduction Labs; 1:2500), HRP-conjugated goat anti-mouse IgG (1:50,000) and ECL reagent is shown. Lane 6 contains the negative control. (C) Following the transfer, the gel was stained with Gelcode Blue to visualize total protein loading of bait protein.
Figure 3. Interactions between eNOS from an endothelial cell lysate and bacterially expressed 6xHis-AL or 6xHis-AS. Bacterially expressed 6xHis-hAL, 6xHis-bAS or rCyB5R-4xHis bound to Ni-NTA agarose was used as the bait protein. Endothelial cells were left in the basal state (basal) or stimulated with 10 µM bradykinin plus 20 µM sodium orthovanadate (BkV). After treatment for 90 minutes, endothelial cell lysates were prepared and used as the prey protein. Proteins were separated by SDS-PAGE under reducing conditions on 4-15% Tris-HCl gels. (A) An immunoblot result of eNOS detected with anti-eNOS monoclonal antibody (BD Transduction Labs; 1:2500), HRP-conjugated goat anti-mouse IgG (1:50,000) and ECL reagent is shown. Lanes 1-2 contains 3% endothelial cell lysate input proteins. Lanes 7-8 contain the negative controls. (B) Following the transfer, the gel was stained with Gelcode Blue to visualize total protein loading of bait protein.
Figure 4. Interactions between caveolin-1 from an endothelial cell lysate and bacterially expressed 6xHis-AL or 6xHis-AS. Bacterially expressed 6xHis-hAL, 6xHis-bAS or rCyB5R-4xHis bound to Ni-NTA agarose was used as the bait protein. Endothelial cells were left in the basal state (basal) or stimulated with 10 µM bradykinin plus 20 µM sodium orthovanadate (BkV). After treatment for 90 minutes, endothelial cell lysates were prepared and used as the prey protein. Proteins were separated by SDS-PAGE under reducing conditions on 4-15% Tris-HCl gels. (A) An immunoblot result of caveolin-1 detected with anti-caveolin-1 monoclonal antibody (BD Transduction Labs; 1:1000), HRP-conjugated goat anti-mouse IgG (1:50,000) and ECL reagent is shown. Lanes 5-6 contain the negative controls. (B) Following the transfer, the gel was stained with Gelcode Blue to visualize total protein loading of bait protein.

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<td>rCyB5R-4xHis (bait)</td>
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B

Gelcode Blue stained gel after transfer
Literature cited


McDonald KK, Zharikov S, Block ER, Kilberg MS. A caveolar complex between the cationic amino acid transporter 1 and endothelial nitric-oxide synthase may explain the "arginine paradox". J Biol Chem 1997; 272 (50):31213-6.


Paper III:
Endothelial Nitric Oxide Production is Tightly Coupled to the Citrulline-NO Cycle
by
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Running title: Citrulline-NO cycle
Abstract

Nitric oxide (NO) is an important vasorelaxant produced along with L-citrulline from L-arginine in a reaction catalyzed by endothelial nitric oxide synthase (eNOS). Previous studies suggested that the recycling of L-citrulline to L-arginine is essential for NO production in endothelial cells. However, there is no direct evidence demonstrating the degree to which the recycling of L-citrulline to L-arginine is coupled to NO production. We hypothesized that the amount of NO formed would be significantly higher than the amount of L-citrulline formed due to the efficiency of L-citrulline recycling via the citrulline-NO cycle. To test this hypothesis, endothelial cells were incubated with $[^{14}\text{C}]-\text{L-arginine}$ and stimulated by various agents to produce NO. The extent of NO and $[^{14}\text{C}]-\text{L-citrulline}$ formation were simultaneously determined. Under basal and stimulated conditions, NO exceeded L-citrulline on the order of 8 to 1, respectively. As further support, $\alpha$-methyl-DL-aspartate, which specifically inhibits L-citrulline recycling, also inhibited NO production in a dose-dependent manner. The results of this study provide evidence for the essential and efficient coupling of L-citrulline recycling, via the citrulline-NO cycle, to endothelial NO production.

Key words: nitric oxide; L-citrulline; L-arginine; endothelial nitric oxide synthase; argininosuccinate synthase
Introduction

Endothelial nitric oxide synthase (eNOS) plays a key role in vasoregulation through the conversion of L-arginine to L-citrulline and the vasorelaxant, nitric oxide (NO). Impaired production of NO associated with hypertension, heart failure, hypercholesterolemia, atherosclerosis, and diabetes may be due to disruption of appropriate regulation of endothelial nitric oxide synthase (eNOS) at several different levels, including substrate availability. The availability of extracellular L-arginine appears to be “rate-limiting” for NO production (Shen et al. 2005; Van Geldre et al. 2002) even though normal intracellular concentrations are well in excess of the reported \( K_m \) (~3 µM) for L-arginine (Hardy et al. 2002; Pollock et al. 1991). This phenomenon has been termed the “arginine paradox” (Flam et al. 2001; Hardy & May 2002; Vukosavljevic et al. 2006). Intracellular L-arginine levels have been reported to range from 0.1 to 0.8 mM (Hardy & May 2002; Shen et al. 2005; Van Geldre et al. 2002), indicating that the bulk of intracellular L-arginine may not be available for conversion to NO. We hypothesized that the other product of the eNOS-catalyzed reaction, L-citrulline, is efficiently recycled via the enzymes of the citrulline-NO cycle, in a tightly coupled process, to the substrate, L-arginine, effectively segregating the L-arginine directed to NO production from bulk intracellular L-arginine. Figure 1 shows the regeneration of L-arginine from L-citrulline in the citrulline-NO cycle. Argininosuccinate synthase (AS) converts L-citrulline to argininosuccinate (Xie et al. 2000). Argininosuccinate is converted to L-arginine by argininosuccinate lyase (AL). The L-arginine so formed is then converted to NO by the action of eNOS to complete the cycle. AS catalyzes the rate-limiting step in the conversion of L-citrulline to L-arginine.

Because eNOS and the cationic amino acid transporter 1 (CAT1), responsible for most of the transport of extracellular L-arginine into endothelial cells, are co-localized in caveolae, it has been widely accepted that this transporter provides the L-arginine directed to NO production (McDonald et al. 1997). However, Li et al. recently showed that endothelial NO production is enhanced through protein-protein interaction between eNOS and CAT1, but L-arginine transport by CAT1 was not required for the observed
enhancement, suggesting that CAT 1 is important for endothelial NO production, but not for providing the L-arginine directed to NO production (Li et al. 2005).

In contrast, several reports have demonstrated that NO production is limited by the capacity to regenerate L-arginine from L-citrulline via the citrulline-NO cycle, particularly under stimulated conditions (Flam et al. 2001; Shen et al. 2005; Su et al. 1995; Van Geldre et al. 2002; Xie et al. 1997; Xie et al. 2000). Amongst the early evidence in support of an important role for the citrulline-NO cycle in endothelial NO production was our observation that extracellular L-citrulline was as effective as L-arginine in stimulating NO production. Even in medium containing saturating levels of L-arginine, L-citrulline stimulated endothelial NO production (Flam et al. 2001). Because extracellular L-citrulline levels had no effect on bulk intracellular L-arginine levels, we concluded that L-arginine regeneration from L-citrulline plays a role in directly providing the L-arginine for NO production in stimulated endothelial cells rather than via conversion to intracellular L-arginine. Similarly, Wu et al. (Wu et al. 1993), showed that synthesis of L-arginine from L-citrulline was stimulated by addition of exogenous L-citrulline. Other evidence supporting an important role for the citrulline-NO cycle in NO production includes the demonstration that overexpression of AS in vascular smooth muscle cells dramatically enhanced the capacity of transfected cells to produce NO even in the presence of excess extracellular L-arginine (Xie & Gross 1997; Xie et al. 2000), leading to the conclusion that the capacity to recycle L-citrulline to L-arginine is “rate-limiting” to NO production. Su et al. (Su & Block 1995) arrived at a similar conclusion, showing that hypoxia inhibited both the induction of AS by endotoxin in pulmonary artery endothelial cells as well as the production of NO, independent of extracellular L-arginine levels.

Recently, Shen et al. (Shen et al. 2005) demonstrated that the AS inhibitor, α-methyl-DL-aspartic acid (MDLA), inhibited NO production in calcium ionophore stimulated endothelial cells, indicating that the recycling of L-citrulline to L-arginine is important for endothelial NO production. Also, gene expression studies demonstrated significant and coordinate upregulation of endothelial AS gene expression in response to fluid shear stress stimulation of NO production (McCormick et al. 2001).
extracellular L-arginine was not limiting in these studies, the authors concluded that a prerequisite for shear stress induced NO production, in the absence of synthesis of additional eNOS, was an increase in L-arginine regeneration via increased AS expression (McCormick et al. 2001). The essential role of AS and the citrulline-NO cycle in endothelial NO production was further reinforced by our recent demonstration of a direct connection between knockdown of AS expression by AS siRNA and reduced NO production (Goodwin et al. 2004).

The enzymes, AS and AL, have been most extensively studied in liver, where they function in the urea cycle to dispose of excess nitrogen as urea. Clearly the functional role of AS and AL is different in endothelial cells than in liver. Previous studies from our laboratory (Pendleton et al. 2002; Pendleton et al. 2005) and others (Cohen et al. 1996) suggested that regulation of AS and localization of AS and AL are also different in liver than in endothelial cells. In liver, AS and AL localize with mitochondria (Cohen & Kuda 1996) to complete the urea cycle. In endothelial cells, we demonstrated that AS and AL co-localize with eNOS in the caveolae fraction of the plasma membrane (Flam et al. 2001). AS has also been shown to respond to metabolite signaling differently in the two tissues. Glutamine induces AS activity in liver tissue (Quillard et al. 1996), but may interfere with AS activity in endothelial cells (Sessa et al. 1990). We found no difference in the primary structure of hepatic and endothelial AS, but in endothelial cells there are additional forms of AS mRNA, with extended upstream open reading frames, that play a role in controlling expression of endothelial AS (Pendleton et al. 2002).

We propose that differences in function, co-localization and regulation of AS relate to the structure/function relationships of a caveolae-localized “nitric oxide synthase/L-arginine regeneration complex”, which in endothelial cells, comprises the citrulline-NO cycle. Herein we report the first direct evidence for the operation of the citrulline-NO cycle through the simultaneous measurement of NO production (as nitrite) and apparent L-citrulline production (as conversion of $[^{14}\text{C}]-\text{L-arginine}$ to $[^{14}\text{C}]-\text{L-citrulline}$) (Hardy & May 2002). The magnitude of the apparent ratio of endothelial NO to L-citrulline produced is an indicator of the degree of coupling of the citrulline-NO cycle.
cycle to endothelial NO production.
Experimental Procedures

Cell culture and reagents – Bovine aortic endothelial cells (BAEC) were grown to confluence in 12-well plates in Dulbecco’s modification of Eagle’s medium (DMEM) containing 10% fetal bovine serum (fbs), 100 IU penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B at 37°C in a 5% CO₂ incubator. On the day of the experiment, BAEC were rinsed in Hanks’ balanced salt solution, without phenol red, containing 20 mM HEPES (HBSS/HEPES). HBSS/HEPES containing 330 µM L-arginine plus 100 µM (S)-(2-Boronoethyl)-L-cysteine hydrochloride (BEC-HCl) was added to each well. After 35 min, the medium was removed and HBSS/HEPES containing 3.0 µM [U-¹⁴C]-L-arginine monohydrochloride (specific activity 305 mCi/mmol, GE Healthcare) was added to each well. Cells were treated with 10 µM bradykinin plus 20 µM sodium orthovanadate for 90 min, in the absence of fbs. For the inhibition studies, cells were treated with an AS inhibitor, α-methyl-DL-aspartic acid (MDLA). For the arginine-deprivation experiments, cells were incubated in a synthetic DMEM plus 10% fbs lacking L-arginine, except for the small contribution of L-arginine by the serum, for 48 h prior to the start of the experiment. All cells were treated with BEC-HCl, a competitive inhibitor of arginases I and II prior to and during the stimulation experiments to prevent conversion of L-arginine to L-ornithine. To inhibit eNOS activity, the NOS inhibitor, N⁵-nitro-L-arginine methylester (L-NAME; 2 mM), was added to cells 35 min prior to and during the stimulation experiments. DMEM, HBSS, HEPES and trypan blue were obtained from Mediatech, Inc. Fetal bovine serum was obtained from HyClone. Bradykinin, L-arginine and MDLA were obtained from Sigma-Aldrich. Sodium orthovanadate and A23187 were obtained from Alexis Biochemicals. BEC-HCl was obtained from EMD Biosciences.

Measurement of cell viability – BAEC were washed with HBSS/HEPES and HBSS/HEPES containing 330 µM L-arginine was added to each well for 60 min. Following this, the medium was removed and HBSS/HEPES containing 330 µM L-arginine, 10 µM bradykinin and 20 µM sodium orthovanadate was added to each well with increasing concentrations of MDLA up to 8 mM. Following a 135 min incubation,
the medium was removed, cells were trypsinized from the plates and cell viability was assessed using trypan blue exclusion counting.

*Measurement of endothelial NO production* – After addition of HBSS/HEPES plus agonists and inhibitors, a portion of the medium was removed from each well for NO determination (as nitrite). At the end time point, another sample of medium was removed for NO determination (as nitrite). L-NAME-inhibitable nitrite production was measured and calculated according to the method of Misko *et al.* (Misko *et al.* 1993). 2,3-Diaminonaphthalene used to quantitate nitrite production was obtained from Sigma-Aldrich (Dejam *et al.* 2004; Kleinhenz *et al.* 2003; Lundberg *et al.* 2005; Misko *et al.* 1993). Samples were read in a Jasco FP-770 spectrofluorometer (excitation = 365 nm; emission = 409 nm), and a standard curve was generated from sodium nitrite standards. As a test of the fluorometric assay, nitrite production in duplicate samples was determined by an alternate method using the Griess assay (Promega). Samples were read in a Beckman DU640B spectrophotometer at 540 nm. Essentially the same results were obtained with either method but the fluorometric method was routinely used because of its greater sensitivity. NOx production was also determined using a nitrate/nitrite colorimetric assay kit (Cayman Chemical Company). Nitrate was reduced to nitrite by nitrate reductase. Following this reduction, nitrite and NOx were measured in all samples using the Griess reagent and samples were quantitated at 540 nm in a µQuant spectrophotometric platereader (Biotek). Standard curves were generated from sodium nitrite and sodium nitrate standards.

NO has a half-life of approximately five sec and, in the presence of water and molecular oxygen, quickly converts to nitrite, a stable end-product. Nitrite can be measured using the fluorescent 2,3-diaminonaphthalene assay (DAN assay) where 2,3-diaminonaphthalene reacts with a nitrosonium cation forming the fluorescent product, 1H-naphthotrazole (Kleinhenz *et al.* 2003; Misko *et al.* 1993). Nitrite can also be measured using the spectrophotometric Griess assay (Promega) where nitrite reacts with sulfanilic acid and N-(1-naphthyl)ethylenediamine to form a purple derivative measurable with a spectrophotometer. Nitrite produced from duplicate samples using either the DAN or Griess assays was comparable for both assays and the fold-activation
observed following exposure of cells to the calcium ionophore A23187 or to bradykinin and sodium orthovanadate was the same (data not shown).

Nitrate is also a possible degradation product of NO, in addition to nitrite. Therefore, nitrate was converted to nitrite by nitrate reductase in order to measure total NOx (nitrate plus nitrite) under basal and stimulated conditions. We found approximately equimolar quantities of nitrite and nitrate under both basal and stimulated conditions (~55% nitrate; ~45% nitrite) and the fold stimulation observed was the same whether measuring total NOx or nitrite alone. Samples were measured by both the fluorometric assay and the Griess method for nitrite. Thus, quantitation of nitrite alone accurately reflects the amount of endothelial NO produced, in agreement with previous reports (Dejam et al. 2004; Kleinbongard et al. 2006; Kleinbongard et al. 2003; Lauer et al. 2001; Lundberg & Weitzberg 2005; Misko et al. 1993). It should be noted, however, that the apparent ratios of NO (as nitrite) to L-citrulline produced are minimal estimates and are probably as much as two fold higher if total NOx was used as a measure of NO produced.

Measurement of endothelial L-citrulline production – At the end of the reaction, ice cold 100% methanol was added to each well to lyse the cells prior to measurement of the extent of conversion of $[^{14}\text{C}]-\text{L-arginine}$ to $[^{14}\text{C}]-\text{L-citrulline}$ (Hardy & May 2002). The sample was mixed with AG 50W-X8, sodium form, cation exchange resin (Bio-Rad Laboratories) equilibrated in stop buffer (50 mM HEPES, pH 5.5, 5 mM EDTA). The samples were transferred to spin-cups (Costar Spin-X, 0.45 µm) and centrifugation was carried out for 2 minutes at 14000g. The flow-through was concentrated by vacuum centrifugation and a portion of the flow-through was retained for analysis by reverse-phase HPLC (Antoine et al. 1999; Tcherkas et al. 2001). Samples were precolumn derivatized with o-phthaldialdehyde reagent solution (Sigma-Aldrich) and then separated isocratically on a System Gold HPLC system with 126 solvent module and 166 UV detector (Beckman-Coulter) using a C18 small pore, 250 mm x 4.6 mm 201SP54 column (Grace VydaC) with column guard. Fractions were collected and mixed with Cytoscint (MP Biomedical) and counts were determined by liquid scintillation counting in an
LS6500 (Beckman-Coulter). L-NAME-inhibitable L-citrulline production was calculated from the L-citrulline peak as determined by reverse-phase C18 column separation.

To test the validity of the ratios observed for apparent production of NO relative to production of L-citrulline by endothelial cells, we determined the ratios of these two products formed in an in vitro assay using recombinant eNOS and $[^{14}\text{C}]$-L-arginine. Under these in vitro conditions there would be no recycling of L-citrulline to L-arginine and no dilution of products by endogenous metabolites. The apparent ratio of NO (as nitrite) to L-citrulline produced was approximately 2, indicating that the ratios observed for the cellular assays are valid.

Intracellular L-arginine concentrations under normal and L-arginine deprived culture conditions – Following the 48 h L-arginine deprivation of BAEC, the cells were rinsed five times with HBSS/HEPES. Ice cold 100% methanol was added to each well to lyse the cells prior to measurement of intracellular L-arginine concentrations. Samples were collected and removed to spin-cups to remove particulate matter. Following precolumn derivatization with o-phthaldialdehyde reagent solution, each sample was injected onto a C18, 250 mm x 4.6 mm 201SP54 column as described previously for L-arginine separation and fractions were collected. The collected fractions were removed to a 96-well black plate (Greiner) and fluorescence was measured using a BMG Labtecnologies Fluostar Galaxy platereader (excitation = 320 nm; emission = 360 nm). L-arginine standards were also analyzed in the same manner. The area under the curve (AUC) for L-arginine was calculated using SigmaPlot 8.0 and was used to calculate intracellular L-arginine concentrations.

L-citrulline percent recovery from cation exchange resin – Samples were analyzed to calculate the amount of L-citrulline flow-through from the cation exchange resin. In vitro assay tubes were prepared with 50 mM HEPES, pH 7.4, 3 µM tetrahydrobiopterin, 1 µM flavin adenine dinucleotide, 1 µM flavin mononucleotide, 1.5 µM calmodulin, 1 mM calcium chloride, 0.1 mM dithiothreitol, 312 µM NADPH, 3 µM L-arginine, 0.2 µM L-[2,3,4,5-$^3$H]-arginine monohydrochloride (GE Healthcare), 75 µM L-[ureido-$^{14}$C]-citrulline (PerkinElmer Life and Analytical Sciences) and either 44 µg heat-inactivated bovine recombinant eNOS or recombinant protein buffer (50 mM HEPES, pH 7.4, 10%
glycerol, 5 mM CHAPS and 100 µM dithiothreitol). The reaction tubes were incubated at 37°C for 15 min and the reaction was stopped with 1.5 mM EGTA. A portion of the reaction mixture was retained for liquid scintillation counting and separation of L-arginine and L-citrulline by reverse-phase HPLC. The remainder of the sample was mixed with cation exchange resin and processed as described in the measurement of endothelial L-citrulline production section. A portion of the flow-through was retained for liquid scintillation counting and separation of L-arginine and L-citrulline by reverse-phase HPLC. Samples were analyzed as described in the measurement of endothelial L-citrulline production section. L-citrulline percent recovery from the reaction mixture and flow-through samples was calculated from the L-citrulline peak as determined by reverse-phase C18 column separation. The mean percent recovery ± SEM from two independent experiments performed in triplicate was 82.6 ± 2.2.

$[^{14}\text{C}]-\text{L-citrulline radiospecific activity} – \text{BAEC were treated for 90 min with} [^{14}\text{C}]-\text{L-arginine in Hanks/HBSS. Samples were treated as detailed in the measurement of endothelial L-citrulline production section. Following precolumn derivatization with o-phthalaldialdehyde reagent solution, each sample was injected onto a C18, 250 mm x 4.6 mm 201SP54 column as and fractions were collected for fluorescence detection and liquid scintillation counting. The peak corresponding to L-citrulline from fluorescence detection and $[^{14}\text{C}]-\text{L-citrulline from liquid scintillation counting was used to calculate intracellular L-citrulline radiospecific activity in µCi/µmol. The calculated radiospecific activity of the $[^{14}\text{C}]-\text{L-citrulline product after cell lysis was used to calculate the amount of} [^{14}\text{C}]-\text{L-citrulline formed in endothelial cells. Because the calculated radiospecific activities also include some endogenous L-citrulline, the calculated amounts of} [^{14}\text{C}]-\text{L-citrulline formed may be an over-estimation in relation to NO formation.}

Statistics – The presented data were calculated from a minimum of three independent experiments. Duplicate wells were tested in each experiment. Results from representative experiments are expressed as the mean ± SEM. Statistical analysis was performed using an unpaired t test (SPSS 14.0 for Windows). A value of $p<0.05$ was considered statistically significant.
Results

Comparative assays for NO and L-citrulline production in endothelial cells – Previous work from our laboratory, and others, suggested that the recycling of L-citrulline to L-arginine via the citrulline-NO cycle plays an essential role in providing L-arginine for endothelial NO production (Goodwin et al. 2004). However, there has been no direct demonstration that NO production is tightly coupled to the citrulline-NO cycle. Thus, based on previous work, we proposed that the L-arginine used by eNOS is provided by the combined action of AS and AL, as components of the citrulline-NO cycle. In the current study, we provide direct evidence for this hypothesis. The amounts of NO and L-citrulline produced were simultaneously quantitated by measuring the formation of nitrite, a stable end-product of NO and molecular oxygen in aqueous solutions (Dejam et al. 2004; Kleinhenz et al. 2003; Lundberg & Weitzberg 2005; Misko et al. 1993), and the conversion of $^{14}\text{C}$-L-arginine to $^{14}\text{C}$-L-citrulline (Hardy & May 2002). If L-citrulline recycling plays no role, or only a minor role in NO production, the apparent amounts of NO and L-citrulline produced should be approximately the same when measured under identical conditions. If, however, L-citrulline recycling provides L-arginine for NO production, the apparent amount of NO produced should be significantly greater than the amount of L-citrulline produced due to the efficient recycling of L-citrulline to L-arginine as part of the citrulline-NO cycle.

Endothelial cells can be stimulated to produce NO by bradykinin or other physiological effectors that signal an increase of intracellular calcium thereby leading to activation of eNOS. Calcium ionophores, such as A23187, bypass the receptor-mediated pathway by increasing available calcium for eNOS activation. We previously showed that the tyrosine phosphatase inhibitor, sodium orthovanadate, further enhanced stimulation of endothelial NO production (Hellermann et al. 2000). As shown in Figure 2, the apparent amount of NO formation is much greater than the apparent amount of L-citrulline formation under either basal or stimulated conditions in cultured endothelial cells. Moreover, the apparent ratio of NO to L-citrulline produced under basal and stimulated (bradykinin and sodium orthovanadate) conditions was approximately 8:1 (Table 1). These results indicate that the recycling of L-citrulline to L-arginine and
subsequent conversion of this L-arginine to NO is very efficient under all conditions. These results also have important implications for the measurement of cellular eNOS activity, indicating that measurements based on L-citrulline production alone may lead to a substantial underestimation of cellular eNOS activity and the degree of stimulation. It should be noted that the calculated ratios of NO to L-citrulline produced represent minimum estimates because NO is estimated as nitrite (does not include nitrate) and L-citrulline estimates are based on radiospecificity measurements that include some endogenous cold L-citrulline. Thus, NO is probably under-estimated, while L-citrulline is probably over-estimated.

Inhibition of NO production by inhibition of AS – In a previous study we showed that selective reduction of endothelial AS expression by transfection of endothelial cells with AS siRNA also reduced the capacity of these cells to produce NO, supporting an essential role for AS in NO production (Goodwin et al. 2004). In the present study, we confirm and extend these results through the use of the inhibitor, α-methyl-DL-aspartic acid (MDLA), which is specific for AS (Shen et al. 2005). Assessment of cell viability using trypan blue exclusion of stimulated BAEC with up to 8 mM MDLA indicated there was no significant cell death compared to stimulated cells alone (data not shown). As shown in Figure 3, the AS inhibitor, MDLA inhibited NO and L-citrulline production in stimulated endothelial cells in a dose dependent manner. However, even when NO production was substantially inhibited (~80%), the ratio of NO (as nitrite) to L-citrulline produced was still high (~7:1), indicating a direct correlation between NO production and AS inhibition and further supporting the tight coupling of the L-citrulline-NO cycle to NO production. Thus, inhibition of AS does not cause an uncoupling of endothelial NO production and the citrulline-NO cycle as indicated by the relatively constant ratio of NO to L-citrulline produced.

Effect of L-arginine deprivation – Intracellular L-arginine concentrations were calculated based on an endothelial cell volume of 1.02 pL/cell (Baydoun et al. 1990; Rubin et al. 1989). The average intracellular L-arginine concentration of normal BAEC versus 48 h L-arginine deprived BAEC was 1.76 to 0.36 mM, respectively. This
corresponds to an 80% decrease in intracellular L-arginine levels following 48 h L-arginine deprivation (Mann et al. 2003).

If endothelial NO production is dependent on the bulk intracellular source of L-arginine, the apparent extent of formation of NO should be reduced in L-arginine deprived cells. As shown in Figure 4, there was only a modest decrease in NO production in L-arginine deprived cells compared to the extent of L-arginine depletion in these cells. Although L-arginine deprivation may result in multiple effects that are beyond the scope of this study, these results support the hypothesis that there is a separate and distinct cellular source of L-arginine substrate directed to endothelial NO production, separate from the bulk intracellular source of L-arginine, consistent with earlier observations that led to the “arginine paradox” (Hardy & May 2002; Hecker et al. 1990; Wu & Meining 1993).
Discussion

The present results provide direct evidence for an efficient and essential coupling of the citrulline-NO cycle to endothelial NO production. These results support a growing body of evidence for the key role of this cycle in NO production since its original discovery by Vane and coworkers in 1990 (Hecker et al. 1990; Sessa et al. 1990). However, the functional importance of this cycle, to provide L-arginine for NO production, was not fully appreciated. This may be due to assumptions regarding alternative sources of L-arginine, including intracellular L-arginine pools and/or the CAT1 transport system which transports most of the intracellular L-arginine. As discussed in the Introduction, the CAT1 transport system may play an important role in endothelial NO production, but not through provision of L-arginine for NO production (Li et al. 2005).

L-Arginine has multiple metabolic precursor functions other than serving as a precursor of NO. This may explain the mixed, and sometimes adverse, effects of L-arginine supplementation to improve vascular function (Bode-Boger 2006). L-Citrulline, in contrast, has only one known precursor metabolic function, conversion to argininosuccinate in either the hepatic urea cycle, renal arginine synthesis, or recycling to L-arginine via the citrulline-NO cycle. Thus, L-citrulline can serve as a precursor of L-arginine directed to NO production without perturbing other metabolic functions associated with L-arginine metabolism. L-arginine dedicated to NO synthesis and provided by the citrulline-NO cycle thus functions to segregate L-arginine directed to NO production sparing bulk intracellular L-arginine for other metabolic roles. The present results, together with previous results from our laboratory (Flam et al. 2001; Goodwin et al. 2004; Solomonson et al. 2003), and others (Braissant et al. 1999; Daniel et al. 2000; Lee et al. 2002; Shuttleworth et al. 1995; Su et al. 1999; Van Geldre et al. 2002), provide a unique view and understanding of endothelial NO production, showing that a caveolar complex involved in substrate (L-arginine) regeneration via a tightly coupled citrulline-NO cycle must be functional to sustain NO production and to maintain cell viability.

Endothelial NO production is very important for vasoregulation and other physiological functions. Adverse effects, and disease states, can be due to either over- or
under-production of NO by endothelial cells. Consistent with the physiological importance of this system, there are several different levels of control, including reversible phosphorylation and other post-translational modifications, protein-protein interactions, cellular translocation, and substrate or cofactor availability. The results of the present study, indicating an efficient and essential coupling of L-citrulline recycling to endothelial NO production, suggest an additional level of control that may provide a unique therapeutic target for modulating endothelial NO production.
Acknowledgements

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List of Abbreviations

AS: Argininosuccinate synthase
BEC-HCl: (S)-(2-Boronoethyl)-L-cysteine hydrochloride
eNOS: Endothelial nitric oxide synthase
L-NAME: N°-nitro-L-arginine methylester
MDLA: α-methyl-DL-aspartic acid
Table 1. NO (as nitrite) and L-citrulline produced under basal and stimulated conditions. BAEC were treated and samples were collected for NO (as nitrite) and L-citrulline production as described in methods. The results shown are the mean ± SEM from six independent experiments. Statistical analyses comparing basal and stimulated (10 μM bradykinin and 20 μM sodium orthovanadate) nitrite and L-citrulline production were performed using an unpaired t test. *p<0.01 was considered statistically significant.

<table>
<thead>
<tr>
<th></th>
<th>NO (nitrite) (pmol/minute/1x10⁶ cells)</th>
<th>L-Citrulline (pmol/minute/1x10⁶ cells)</th>
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<tbody>
<tr>
<td>Basal</td>
<td>0.39 ± 0.06</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>Stimulated</td>
<td>7.55 ± 0.81 *</td>
<td>1.01 ± 0.23 *</td>
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Figure 1. The L-citrulline-NO cycle. L-arginine is converted to nitric oxide and L-citrulline by endothelial nitric oxide synthase. L-citrulline and aspartate are then converted to argininosuccinate by argininosuccinate synthase. Argininosuccinate is converted to fumarate and L-arginine by argininosuccinate lyase.
Figure 2. NO and L-citrulline production in endothelial cells. BAEC were treated and samples were collected for NO (as nitrite) and L-citrulline production as described in methods. The results shown are the mean ± SEM from six independent experiments. Statistical analysis was performed using an unpaired t test. *$P<0.01$ was considered statistically significant.
Figure 3. Inhibition of endothelial NO and L-citrulline production by α-methyl-DL-aspartic acid (MDLA). BAEC were treated and samples were collected for NO (as nitrite) and L-citrulline production as described in methods. The results shown are the mean ± SEM from three independent experiments.
Figure 4. Effect of L-arginine deprivation on endothelial NO production in endothelial cells. BAEC were treated and samples were collected for NO (as nitrite) production as described in methods. The results shown are the mean ± SEM from six independent experiments in the L-arginine sufficient state (+arg) and three independent experiments in the L-arginine deprived state (-arg). Statistical analyses were performed using an unpaired t test. **P<0.05 was considered statistically significant.
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Paper IV:
Endothelial Nitric Oxide Production is Affected by Glucosamine Treatment
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Objectives — Nitric oxide (NO) production by endothelial nitric oxide synthase (eNOS) as part of the citrulline-NO cycle is essential for cardiovascular health. However, impairment of citrulline-NO cycle proteins by dynamic post-translational protein modifications, like O-linked β-N-acetylglucosamine (O-GlcNAc) modifications at serine/threonine residues may lead to impaired signaling in the pathway. This study examines the effects of glucosamine treatment on NO production and O-GlcNAc modifications of endothelial cell proteins.

Methods and Results — Glucosamine enters the hexosamine biosynthetic pathway after the rate limiting step catalyzed by glutamine:fructose-6-phosphate transferase. Increased concentrations of glucosamine lead to increased uridine diphospho-N-acetylglucosamine (UDP-GlcNAc) production, which is added to proteins by O-GlcNAc transferase. Basal or stimulated endothelial cells were treated for 20-26 hours with increasing concentrations of glucosamine. Basal NO production was elevated after chronic glucosamine treatment while glucosamine treatment blunted calcium agonist-stimulated NO production in endothelial cells. In stimulated endothelial cell lysates, argininosuccinate synthase and eNOS proteins were elevated in glucosamine treated cells. There was a slight increase in total O-GlcNAc protein modifications in glucosamine treated, stimulated endothelial cell lysates as assessed by immunoblotting.

Conclusions — These data suggest that after chronic glucosamine treatment, elevated basal NO production results from O-GlcNAc modifications of citrulline-NO cycle proteins. After chronic glucosamine treatment in stimulated endothelial cells, decreased eNOS activity caused by O-GlcNAc modifications leads to a reduction in endothelial NO production.

Key words: nitric oxide; glucosamine; O-linked β-N-acetylglucosamine; endothelial nitric oxide synthase; argininosuccinate synthase
Introduction

Protein modification is a mechanism to regulate enzyme activity, localization and signaling pathways and is typically achieved by phosphorylation and dephosphorylation on serine, threonine or tyrosine residues. These events are catalyzed by kinases for phosphorylation and phosphatases for dephosphorylation. In 1984, Torres and Hart found another mode of dynamic and regulated protein modification (Torres et al. 1984). The O-linked β-N-acetylglucosamine (O-GlcNAc, Appendix D) moiety added to or removed from serine or threonine residues regulates signaling pathways, enzyme activity and localization. This type of protein modification is distinct from protein phosphorylation and dephosphorylation, although adjacent sites can regulate the addition of either O-GlcNAc or phosphate (Kamemura et al. 2002).

The uridine diphospho-N-acetylglucosamine (UDP-GlcNAc) moiety is the product of the hexosamine biosynthetic pathway (Figure 1) and can act as a nutrient sensor (Slawson et al. 2003; Wells et al. 2003). Approximately 2-5% of glucose entering the cell is directed to the hexosamine biosynthetic pathway where it is converted to glucose-6-phosphate. Glucose-6-phosphate is converted to fructose-6-phosphate which is converted to glucosamine-6-phosphate by glutamine:fructose-6-phosphate amidotransferase (GFAT) in the rate limiting step. Glucosamine (Appendix D), synthesized from fructose-6-phosphate and glutamine, enters the hexosamine biosynthetic pathway after the GFAT rate limiting step (Figure 1). GFAT is regulated by feedback inhibition by UDP-GlcNAc concentrations within mammalian cells in a competitive manner with respect to fructose-6-phosphate.

Glucosamine induces insulin resistance at lower concentrations than glucose (Vosseller et al. 2002). Incubation of heart and skeletal muscle with high concentrations of glucosamine increases glucosamine-6-phosphate concentrations within the cell and depletes intracellular ATP levels (Buse 2006). Buse et al. also saw changes in gene expression in 3T3-L1 adipocytes incubated with 5 mM glucose, 2.5 mM glucosamine and 0.6 nM insulin as compared to cells incubated with 25 mM glucose and 0.6 nM insulin. These gene expression changes were specific to glucosamine exposure rather than just increased glucose flux through the hexosamine biosynthetic pathway (Buse 2006).
Endothelial cells experience changes in gene expression (e.g. transforming growth factor-beta1 and plasminogen activator inhibitor-1) where these changes may only be seen after exposure to high glucose or glucosamine (Du et al. 2000; Goldberg et al. 2002). Therefore, regulation of the hexosamine biosynthetic pathway by controlling entry of either glucose or glucosamine may be a method of controlling some of the multigenic effects due to type 2 diabetes, insulin resistance and cardiovascular disease (Akimoto et al. 2005).

Nitric oxide (NO) produced by arginine endothelial nitric oxide synthase (eNOS) is important in preventing early atherosclerotic events and later cardiovascular disease since it suppresses CD11/CD18 expression on circulating leukocytes, protecting blood vessels from leukocyte adhesion to endothelial cells (Forstermann et al. 2006). Citrulline, the other reaction product, is converted by argininosuccinate synthase (AS) and argininosuccinate lyase (AL), respectively, to arginine, as the substrate for eNOS. Our laboratory showed that exogenous citrulline in the presence of saturating levels of arginine for eNOS ($K_m \sim 2.9 \, \mu M$) significantly increased NO production in stimulated endothelial cells as compared to endothelial cells incubated with arginine alone. Furthermore, we showed a co-fractionation of citrulline-NO cycle enzymes from endothelial cell caveolae membranes (Flam et al. 2001; Solomonson et al. 2003). Using an antibody against GM130 (golgi matrix protein of 130 kDa), we showed that the caveolae membrane fraction did not contain other membrane proteins. More recently we showed a direct interaction and association of citrulline-NO cycle proteins (Paper II) along with arginine recycling (Solomonson et al. 2003). Consistent with these observations, transcriptional or post-translational protein modifications may affect the ability of citrulline-NO cycle enzymes to effectively recycle citrulline to arginine for endothelial NO production.

O-GlcNAc protein modifications occur on chromatin, transcription factors, (e.g. Sp1 and AP-1), kinases (e.g. casein kinase II and phosphoinositol 3-kinase), heat shock proteins (e.g. hsp60 and hsp70) and eNOS (Anderson et al. 1991; Brasse-Lagnel et al. 2003; Du et al. 2001; Jackson et al. 1988; Kim et al. 2006; Majumdar et al. 2006; Walgren et al. 2003). The O-GlcNAc modification around serine 1173 in bovine eNOS
leads to a decrease in serine 1179 phosphorylation by Akt causing decreased eNOS enzyme activity (Du et al. 2001; Federici et al. 2002). AS, which catalyzes the rate-limiting step of the citrulline-NO cycle has three Sp1 transcription binding sites within the promoter region (Anderson & Freytag 1991). Sp1 is modified by phosphorylation (Jackson et al. 1993) and O-glycosylation (Jackson & Tjian 1988). These modifications change Sp1’s association with transcription binding sites in gene promoter regions causing changes in mRNA and protein expression. In Caco-2 cells, a human intestinal cell line, glutamine and glucosamine increased O-GlcNAc modifications of Sp1 causing greater nuclear translocation and greater Sp1 binding to the AS promoter initiating increased transcription (Brasse-Lagnel et al. 2003). In the rat AL promoter, there is one Sp1 and one nuclear factor-Y (NF-Y) transcription factor binding site (Matsubasa et al. 1994; Takiguchi et al. 1995). Examination of the NF-Y sequence with the YinOYang prediction server shows many potential O-GlcNAc modification sites. Therefore, O-GlcNAc modifications of Sp1 and/or NF-Y may cause a change in the binding of this transcription factor to the AL promoter leading to increased or decreased gene transcription. Thus, O-GlcNAc modifications may function in the control of gene transcription or protein translation in the citrulline-NO cycle. This study examined the effects of glucosamine treatment on NO production in endothelial cells and its effects on citrulline-NO cycle proteins.
Methods

Cell culture and reagents – Bovine aortic endothelial cells (BAEC) and bovine pulmonary artery endothelial cells (BPAEC) were cultured in Dulbecco’s modification of Eagle’s medium (DMEM) containing 10% fetal bovine serum (fbs), 100 IU penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B at 37°C in a 5% CO₂/95% air incubator. Cells were cultured and grown to confluence in 6 or 12-well plates. The cells were rinsed with Hanks balanced salt solution (HBSS) without phenol red, containing 20 mM HEPES. Then DMEM, without serum, containing 0.2 mM L-glutamine and 15 mM HEPES plus or minus 10 μM bradykinin was added to each well. Cells were treated with increasing concentrations of glucosamine for up to 26 hours. Some cells were treated with 2 mM L-NAME, an eNOS inhibitor, 20 μM DON, a glutamine amidotransferase inhibitor or 40 μM PUGNAc, an O-GlcNAcase inhibitor. DMEM, HBSS, HEPES, and L-glutamine were obtained from Mediatech, Inc. Fetal bovine serum was obtained from HyClone. DMEM without phenol red was obtained from Invitrogen. Bradykinin, glucosamine hydrochloride, L-NAME and DON were obtained from Sigma-Aldrich. PUGNAc was obtained from Carbogen (Aarau, Switzerland). See Appendix D for chemical structures.

Assay of endothelial NO production – After addition of DMEM and glucosamine, a portion of the medium was removed from each well for NO determination (as nitrite). At the end time point, another sample of medium was removed for NO determination (as nitrite). Nitrite was measured according to the method of Misko et al. (Misko et al. 1993). 2,3-Diaminonaphthalene used to quantitate nitrite production was obtained from Sigma-Aldrich (Dejam et al. 2004; Kleinhenz et al. 2003; Lundberg et al. 2005; Misko et al. 1993). Samples were read in a Jasco FP-770 spectrofluorometer (excitation = 365 nm; emission = 409 nm), and a standard curve was generated from sodium nitrite standards.

BAEC lysate preparation – After samples of medium were removed for NO determination at the last time point, the plates were placed on ice, medium was removed and 1% SDS and 2x reducing sample buffer (Bio-Rad Laboratories) were added to each well. Cells were scraped from the dish and the cell lysates were heated at 95°C for 10 minutes. Lysates were stored at -80°C until analyzed.
Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting – Endothelial cell lysates were mixed with reducing sample buffer, heated for 5 minutes at 95°C and loaded on a 4-20 % Tris-HCl gradient gel (Life Gels). The proteins were separated using a Mini-Protean II (Bio-Rad Laboratories) following the manufacturer’s instructions. Following SDS-PAGE, the proteins were transferred to Immobilon-P polyvinylidene fluoride (PVDF) membranes (Millipore) using a Mini-Protean transfer system (Bio-Rad Laboratories). For eNOS, AS and GAPDH detection, the membrane was removed and blocked in 5% non-fat dry milk (NFDM) in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 hour at room temperature. Primary antibody, 1:2500 anti-eNOS, mouse monoclonal (BD Transduction Labs); 1:2500 anti-AS, mouse monoclonal (BD Transduction Labs) or 1:1000 anti-GAPDH, mouse monoclonal (Novus Biologicals) prepared in 5% NFDM/TBST was added to the membrane and incubated overnight at 4°C. The membrane was then rinsed with TBST several times. A secondary antibody, peroxidase-conjugated affinity pure goat anti-mouse IgG (Jackson ImmunoResearch Labs), prepared in 5% NFDM/TBST was added to the membrane and incubated for 1 hour at room temperature. The membrane was again washed with TBST and proteins were detected using ECL (GE Healthcare). For O-linked β-N-acetylglucosamine (O-GlcNAc) modified protein detection, the membrane was blocked in Tris-buffered saline containing 0.3% Tween-20 (TBS-HT) for 1 hour at room temperature. Primary antibody, 1:1000 anti-O-GlcNAc, CTD110.6, mouse IgM (Covance), prepared in TBS-HT was added to the membrane and incubated overnight at 4°C. The membrane was then rinsed with Tris-buffered saline containing 0.3% Tween-20, 0.1% sodium deoxycholate, 0.05% SDS and 0.5% Triton X-100 (TBS-D) followed by TBS-HT. A secondary antibody, peroxidase-conjugated affinity pure goat anti-mouse IgM (Jackson ImmunoResearch Labs), prepared in TBS-HT was added to the membrane and incubated for 1 hour at room temperature. The membrane was again washed with TBS-D followed by TBS-HT and proteins were detected using ECL.

Statistics – Results from representative experiments are expressed as the mean ± SEM. The presented data were calculated from a minimum of two independent experiments. Duplicate wells were tested in each experiment. Statistical analysis was
performed using a one-way ANOVA with Dunnett’s 2-tail test or unpaired t-test (SPSS for Windows version 14.0). A value of $P<0.05$ was considered statistically significant.
Results

*Basal NO (as nitrite) produced by BAEC in the presence of high and low glucose and glutamine* – The amount of NO (as nitrite) produced by BAEC in the presence of 5 mM glucose and either low glutamine (0.2 mM) or high glutamine (2 mM) was significantly greater with the low glutamine treatment. NO (as nitrite) produced by BAEC in the presence of 20 mM glucose and either low glutamine (0.2 mM) or high glutamine (2 mM) was greater with the low glutamine treatment. To prevent conversion of glutamine to UDP-GlcNAc, the substrate for O-GlcNAc transferase, BAEC were treated with 50 µM 6-diazo-5-oxo-L-norleucine (DON), a glutamine amidotransferase inhibitor of the rate limiting-step of the hexosamine biosynthetic pathway. Using the DON inhibitor, the amount of NO (as nitrite) produced by BAEC in the presence of 2 mM glutamine and either 5 mM or 20 mM glucose and was increased as compared to cells not treated with the inhibitor (Table 1). This indicates the involvement of glutamine in the hexosamine biosynthetic pathway since DON inhibits the conversion of glutamine and fructose-6-phosphate into the downstream UDP-GlcNAc, the substrate for O-GlcNAc transferase.

O-GlcNAc modifications have been shown to decrease eNOS activity and increase AS translation (Brasse-Lagnel et al. 2003; Du et al. 2001). These data compare with results from Wu et al. who used bovine venular endothelial cells in the presence of 10 mM glucose and showed that high glutamine caused a decrease in NO production in basal endothelial cells and that the effect was reversed by using a GFAT inhibitor to prevent production of UDP-GlcNAc (Wu et al. 2001).

*NO produced by basal BAEC in the presence of glucosamine* – In basal BAEC treated with 0.2 mM L-glutamine, 10 mM glucose and glucosamine, there was a significant dose dependent increase in NO production (as nitrite) at 2.1, 10.5 and 15 mM glucosamine (Figure 2). The concentration of 10 mM glucose in the medium was to ensure sufficient NADPH as a cofactor for eNOS over the 24 hour treatment (Wu et al. 2001). Duplicate experiments were performed using bovine pulmonary artery endothelial cells (BPAEC) to determine if the increase in NO production (as nitrite) was due to
endothelial cell type. The amount of NO (as nitrite) produced by BPAEC under the same conditions was similar to that seen with the BAEC (data not shown).

The dose dependent effects of glucosamine, observed under basal conditions, were in contrast to the results seen by Wu et al. (Wu et al. 2001) who observed a significant decrease in NO production. Fulton et al. showed that the ratio of eNOS to von Willebrand factor, an endothelial marker, was approximately 2.5 times greater in the circumflex artery than the aorta (Fulton et al. 2000). NO production has also been shown to be lower in venous as compared to arterial endothelial cells (Rotmans et al. 2005). Endothelial NOS distribution differs between different endothelial cell types and may even vary within the endothelial cell itself which could explain differences in NO production (Oess et al. 2006). The amount of basal NO (as nitrite) produced by the BAEC in our experiments was approximately 25 times greater than the amount of basal NO (as nitrite plus nitrate) produced by the coronary venular endothelial cells. The differences seen between the two experiments may be due to the source of the endothelial cells and the cells were maintained in different concentrations of glucose.

NO produced by stimulated BAEC in the presence of increasing concentrations of glucosamine – In stimulated BAEC (10 µM bradykinin) treated with 0.2 mM L-glutamine, 10 mM glucose and glucosamine, there was a dose dependent decrease in NO production (as nitrite) at 2 and 8 mM glucosamine (Figure 3). eNOS activity is decreased by O-GlcNAc modifications (Du et al. 2001). AS translation is increased due to O-GlcNAc modifications of Sp1 which binds to response elements in the AS promoter, causing increased translation (Brasse-Lagnel et al. 2003). However, increased AS translation may be unable to overcome eNOS inhibition by O-GlcNAc modifications, especially if the stimulated citrulline-NO cycle is expected to produce more NO than in the basal state.

Glucosamine effects on eNOS protein, AS protein and O-GlcNAc protein modifications in treated endothelial cells – We then looked at the levels of eNOS and AS using protein specific antibodies and also O-GlcNAc modification of proteins using an antibody to detect O-GlcNAc protein modifications in cell lysates from bradykinin-stimulated endothelial cells in the absence and presence (2 mM and 8 mM) of
glucosamine. We found increases in total eNOS and AS proteins upon incubation with 2 mM and 8 mM glucosamine for 26 hours (Figure 4). Also shown is the ratio of band density for eNOS and AS compared to GAPDH, used as a loading control. AS protein levels increased with glucosamine addition as expected. In the presence of high glutamine concentrations, Brasse-Lagnel et al. showed increased UDP-GlcNAc levels, the substrate for O-GlcNAc transferase, leading to increased O-GlcNAc modified Sp1, which translocates to the nucleus and increases AS gene transcription in Caco-2 cells (Brasse-Lagnel et al. 2003). Du et al. did not see a change in total cellular eNOS protein levels upon incubation with high glucose, although they showed an increase in O-GlcNAc modified eNOS protein along with a decrease in phosphorylated (serine 1177) eNOS protein. Our results showed an increase in eNOS protein levels upon incubation with 2 and 8 mM glucosamine. The differences between the two experiments may be due to the source of the eNOS protein. Du et al. transfected a plasmid encoding myc-tagged human eNOS into BAEC and then determined total eNOS protein with antibodies against myc (human eNOS) or eNOS (human and bovine) (Du et al. 2001). In our experiments, total cellular bovine eNOS protein was detected using an antibody against eNOS. Although we saw a decrease in eNOS activity (based on NO production) along with an increase in total cellular eNOS protein, it is possible that this additional eNOS protein was not enzymatically active and therefore would not contribute to endothelial NO production.

Glucosamine addition caused a slight increase in total cellular O-GlcNAc modified proteins in bradykinin-stimulated endothelial cell lysates (Figure 5). Those cells treated with 40 µM PUGNAc, an O-GlcNAcase inhibitor, showed increased O-GlcNAc modified proteins as compared with untreated cells. Our results are in agreement with Du et al. who showed an increase in total cellular O-GlcNAc modified endothelial cell proteins upon incubation with high glucose (Du et al. 2001).
Discussion

Nandi et al. (Nandi et al. 2006) demonstrated a technique to locate O-GlcNAc modified proteins on a global scale for proteomic analysis. They used HeLa cervical adenocarcinoma cells incubated with a peracetylated azido-GlcNAc which was taken up into cells and converted to UDP-azido-GlcNAc. The UDP-azido-GlcNAc was added to proteins that typically experience O-GlcNAc modifications by O-GlcNAc transferase. Following labeling, HeLa cell lysates were prepared and analyzed by nano-HPLC/tandem mass spectrometry. Proteins that showed O-GlcNAc modifications were analyzed further by immunoprecipitation to confirm mass spectrometry results. The authors confirmed O-GlcNAc modifications on many proteins while increasing the known number of O-GlcNAc modified proteins to over 200. Two members of the heat shock protein 90 (hsp90) family, hsp90 alpha and beta, were confirmed to have O-GlcNAc modifications.

Hsp90 was shown to associate with eNOS in endothelial cells by using fusion-tagged caveolin-1 to isolate the proteins from endothelial cell lysates (Gratton et al. 2000). Gratton et al. also showed direct protein-protein associations between recombinant eNOS and purified hsp90. Nandi et al. determined that AS can be O-GlcNAc modified (Nandi et al. 2006).

Under conditions of high glucose, glutamine or glucosamine, alone or in combination, endothelial cell Sp1 becomes more O-GlcNAc modified leading to increased AS promoter binding and increased AS transcription (Anderson & Freytag 1991; Brasse-Lagnel et al. 2003). Nandi et al. showed that AS protein undergoes O-GlcNAc modifications which may change its enzyme activity or ability to associate with proteins involved in endothelial NO generation (Nandi et al. 2006). The increased activity or quantity of AS may be able to overcome the inhibition of eNOS by O-GlcNAc modification under basal conditions pushing the citrulline-NO cycle to produce more NO. However, under calcium agonist stimulated conditions, if eNOS is O-GlcNAc modified and has decreased activity, even endothelial cells having increased AS activity or quantity may be unable to generate NO at the same rate as cells not treated with glucosamine. Additionally, S. Solomon’s laboratory showed that calmodulin gene transcription is turned on by insulin treatment in H-411E liver hepatoma cells (Majumdar
et al. 2006). The calmodulin gene promoter contains three Sp1 binding sites. O-GlcNAc modification of Sp1 causes the modified transcription factor to translocate to the nucleus where it is then deglycosylated and subsequently phosphorylated. The newly phosphorylated Sp1 binds to sites in the calmodulin promoter, increasing gene transcription (Majumdar et al. 2003; Majumdar et al. 2006). It is possible that under high glucosamine concentrations, more calmodulin is expressed. Calmodulin binds to eNOS, displacing caveolin-1, causing dimerization and activation of eNOS. More activated eNOS leads to increased NO production depending on whether or not eNOS has already been O-GlcNAc modified which would decrease its enzyme activity.

In summary, our data demonstrate an increase in NO production by glucosamine in basal endothelial cells and a reduction in NO production by glucosamine in bradykinin-stimulated endothelial cells. The O-GlcNAc modifications of eNOS, AS and possibly AL may play a role in these seemingly contradictory results. Further study into modifications of these citrulline-NO cycle proteins in endothelial cells along with chronic versus acute glucosamine and glutamine exposure under various physiologic states is needed to determine the exact mechanisms causing these observed results.
Acknowledgements

This work was supported by American Heart Association Florida Affiliate Grants 9950864V and 0455228B and the University of South Florida Foundation -- Mary and Walter Traskiewicz Memorial Fund.
Table 1. NO (as nitrite) in nmol/hour/1x10⁶ cells produced by BAEC under basal conditions with high and low glucose. BAEC were treated and samples were collected for NO (as nitrite) as described in methods. The results shown are the mean ± SEM from at least two independent experiments. Statistical analyses comparing low glucose (5 mM) and high glucose (20 mM) nitrite production were performed using an unpaired t test. P<0.05 (*) was considered statistically significant.

<table>
<thead>
<tr>
<th>Glutamine (mM)</th>
<th>Glucose (mM)</th>
<th>Basal NO (N)</th>
<th>Basal NO in presence of DON (50 µM) (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>5.0</td>
<td>14.92 ± 2.39 (7)</td>
<td>4.17 ± 0.98 (2)</td>
</tr>
<tr>
<td>2.0</td>
<td>5.0</td>
<td>2.31 ± 1.74 (2) *</td>
<td>7.84 ± 1.54 (2)</td>
</tr>
<tr>
<td>0.2</td>
<td>20.0</td>
<td>13.27 ± 3.52 (3)</td>
<td>8.14 ± 4.87 (3)</td>
</tr>
<tr>
<td>2.0</td>
<td>20.0</td>
<td>7.91 ± 5.06 (3)</td>
<td>10.79 ± 3.47 (3)</td>
</tr>
</tbody>
</table>
**Figure 1. UDP N-acetylglucosamine pathway.** Glucose-6-phosphate is converted to fructose-6-phosphate which is converted to glucosamine-6-phosphate by glutamine:fructose-6-phosphate amidotransferase (GFAT) in the rate limiting step. Glucosamine-6-phosphate is then converted to UDP-GlcNAc used as the donor sugar for O-GlcNAc modifications on proteins. Glucosamine, synthesized from fructose-6-phosphate and glutamine, enters the hexosamine biosynthetic pathway after the rate limiting step catalyzed by GFAT. GFAT activity is inhibited by 6-diazo-5-oxo-L-norleucine (DON).

![UDP N-acetylglucosamine pathway diagram](image)

- **GFAT** — glucosamine:fructose-6-phosphate amidotransferase (rls: rate limiting step)
- **DON** — 6-diazo-5-oxo-L-norleucine (GFAT inhibitor)
- **UDP-GlcNAc** — UDP N-acetylglucosamine (substrate for O-GlcNAc transferase)
Figure 2. NO production by basal endothelial cells in the presence of glucosamine.

BAEC were treated for 20-26 hours with glucosamine and samples were collected for NO (as nitrite) production as described in methods. The results shown are the mean ± SEM from at least two independent experiments. Statistical analysis was performed using a one-way ANOVA with 2-tail Dunnett t-test. \( P<0.05 \) (*) was considered statistically significant.
Figure 3. NO production by stimulated endothelial cells in the presence of glucosamine. BAEC were treated for 20-26 hours with glucosamine and stimulated with 10 μM bradykinin and samples were collected for NO (as nitrite) production as described in methods. The results shown are the mean ± SEM from a minimum of two independent experiments. Statistical analysis was performed using a one-way ANOVA with 2-tail Dunnett t-test. P<0.05 (*) was considered statistically significant.
Figure 4. Glucosamine effects on eNOS and AS proteins in stimulated endothelial cells. BAEC were treated with glucosamine and stimulated with 10 µM bradykinin (Bk) and samples were collected for protein analysis. Proteins were separated by SDS-PAGE followed by transfer to PVDF membrane for immunoblotting with antibodies against eNOS, AS and GAPDH. The leftmost lane contains prestained protein molecular weight markers. The results shown are representative from two experiments. Density analysis was performed using ImageQuant 5.2 (GE Healthcare) and eNOS and AS protein levels were normalized to GAPDH.
Figure 5. Glucosamine effects on O-GlcNAc protein modifications in crude lysates isolated from stimulated endothelial cells. BAEC were treated with glucosamine and stimulated with 10 µM bradykinin (Bk) and samples were collected for protein analysis. Some cells were also treated with PUGNAc, an inhibitor of O-GlcNAcase. Proteins were separated by SDS-PAGE followed by transfer to PVDF membrane for immunoblotting with an antibody against O-GlcNAc modified proteins. The leftmost lane contains prestained protein molecular weight markers. The results shown are representative from two experiments.
Literature cited


Conclusions

Endothelial cells contain enzymes responsible for the production of endothelial nitric oxide (NO) and for the downstream effects of vasodilation, vascular health and maintenance of cell viability (Cooke 2004; Goodwin et al. 2004). In the healthy endothelium, NO is produced, as part of the citrulline-NO cycle, from arginine (regenerated from citrulline) by the sequential action of three enzymes, endothelial nitric oxide synthase (eNOS), argininosuccinate synthase (AS) and argininosuccinate lyase (AL) as part of the citrulline-NO cycle (Fig. 1). However, underlying diseases or risk factors (e.g. obesity, type 2 diabetes, atherosclerosis, insulin resistance, high-fat diet) cause endothelial dysfunction and decreased NO production due to changes in citrulline-NO cycle proteins and may lead to cardiovascular disease. Unfortunately over the past three decades the prevalence of cardiovascular disease has increased dramatically and it has been reported that one in three Americans suffers from some type of cardiovascular disease (Poirier et al. 2006). Obtaining a greater understanding of the enzymes, reactions and the extracellular stimuli affecting the citrulline-NO cycle may ultimately allow researchers and clinicians to better design therapeutic interventions to restore endothelial function in individuals with compromised NO production.

In this work we show that the enzymes involved in the citrulline-NO cycle supporting endothelial NO generation, eNOS, AS and AL, are functionally associated, reactions are efficiently coupled and that nutrient levels affect the citrulline-NO cycle proteins. First, we show that even in the presence of saturating levels of arginine for eNOS, addition of exogenous arginine or exogenous citrulline causes an increase in endothelial NO production. We also show that eNOS, AS and AL co-fractionate with endothelial caveolae membranes. To further support our hypothesis, we show a direct interaction as well as associations between the enzymes of the citrulline-NO cycle using recombinant proteins, *in vitro* translated proteins and endothelial cell lysates. Next we show, by simultaneously measuring endothelial NO and citrulline production, a coupling
of the reactions involved in the citrulline-NO cycle. Finally, we show that glucosamine which enters the hexosamine biosynthetic pathway past the rate limiting step, causes changes in basal and stimulated endothelial NO production along with changes in some of the citrulline-NO cycle proteins when added to endothelial cell culture in high concentrations.

Shortly after NO was determined to be the endothelial-derived relaxing factor by Furchgott, Ignarro and Murad, who received the Nobel Prize in Physiology and Medicine for their work, Vane and colleagues reported on an interesting phenomenon observed in cultured endothelial cells (Hecker et al. 1990b). The researchers hypothesized that urea cycle enzymes, AS and AL, might be involved in endothelial NO generation. After depleting endothelial cells of arginine, they supplemented the cultures with the urea cycle intermediates, citrulline and argininosuccinate. Arginine-depleted endothelial cells incubated with argininosuccinate did not increase intracellular arginine levels as compared with arginine-depleted cells without argininosuccinate. They determined that argininosuccinate did not increase intracellular arginine levels since it was unable to either diffuse or be transported across the cell membrane. The interesting observation was that the arginine-depleted cells converted 8.4% of $^{14}$C-citrulline into $^{14}$C-arginine over a 60 minute period. Nondepleted cells converted 3% of $^{14}$C-citrulline into $^{14}$C-arginine over a 60 minute period (Hecker et al. 1990b). Vane’s group also found that incubating endothelial cells with glutamine and stimulating in the presence of a calcium ionophore, caused an approximately three-fold increase in citrulline levels. This observation indicated that glutamine caused a buildup of citrulline and inhibited the recycling of citrulline to arginine in endothelial cells (Hecker et al. 1990a; Sessa et al. 1990).

Subsequently, Wu and Meininger looked at arginine regeneration from citrulline in venular and aortic endothelial cells. They determined that in venular endothelial cells, arginine regeneration was not saturated at normal plasma citrulline levels of 50-100 µM up to 500 µM. Wu and Meininger determined that in intact endothelial cells, independent of extracellular arginine concentrations, citrulline is recycled to arginine (Wu & Meininger 1993).
During this time, our laboratory was working on pathways involved in methylarginine generation to regulate eNOS activity. The enzyme, dimethylarginine dimethylamino hydrolase (DDAH), converts N\textsuperscript{G}\text{-methylarginine or N\textsuperscript{G},N\textsuperscript{G}\text{-dimethylarginine, to citrulline and monomethylamine or dimethylamine, respectively. We added citrulline to endothelial cells expecting to inhibit the enzyme DDAH, preventing methylarginine breakdown, which was expected to inhibit eNOS activity and lower endothelial NO production. However, we found that the addition of the citrulline caused an increase in endothelial NO production (Appendix C). We then measured NO production in stimulated endothelial cells in the presence of increasing concentrations of arginine and citrulline. Thirty minutes prior to stimulation with bradykinin, endothelial cells were switched to Dulbecco’s modified Eagle’s medium (DMEM) containing concentrations of arginine or citrulline from 5-500 µM (DMEM normally formulated with 440 µM arginine). After 30 minutes, the cells were stimulated with 10 µM bradykinin and endothelial NO production was measured after 60 minutes. We found a significant increase in NO production in the presence of increasing concentrations of citrulline or arginine from 125-500 µM (Paper I, Figure 1). Next we measured the effect of exogenous arginine or exogenous citrulline on intracellular citrulline or arginine concentrations in bradykinin-stimulated endothelial cells. The endothelial cell medium was switched to DMEM containing arginine or citrulline from 0-1000 µM for 30 minutes prior to bradykinin stimulation. Sixty minutes after bradykinin stimulation, endothelial NO production was determined and the cells were harvested. Intracellular arginine and citrulline concentrations were determined by reversed-phase HPLC. The relative intracellular arginine or citrulline levels (threonine was used as an internal standard) increased proportionally with increasing extracellular arginine or citrulline, respectively. Relative intracellular arginine levels did not change with increasing extracellular citrulline concentrations. Relative intracellular citrulline levels did not change with increasing extracellular arginine concentrations even though there were associated increases in NO production (Paper I, Figure 2). This suggested that citrulline was recycled to arginine (preventing a buildup of citrulline), that extracellular arginine or citrulline could support NO production even though intracellular levels of arginine were
well above the $K_m$ of eNOS for arginine and that exogenous citrulline was able to mimic the effects of exogenous arginine on NO production. Next we determined the effects of exogenous citrulline or exogenous arginine in arginine-depleted DMEM or arginine-supplemented DMEM on bradykinin-stimulated endothelial cells. Endothelial cells were switched to arginine-depleted DMEM containing: (A) 0 µM arginine, (B) 500 µM arginine, (C) 500 µM citrulline, or arginine-supplemented DMEM containing: (D) 440 µM arginine, (E) 940 µM arginine or (F) 440 µM arginine plus 500 µM citrulline. After 30 minutes, the endothelial cells were stimulated with bradykinin for 60 minutes and endothelial NO production was calculated for the different culture conditions. In conditions A-C, exogenous arginine or exogenous citrulline enhanced bradykinin-stimulated endothelial NO production (B and C) over that of stimulated endothelial NO production in arginine-depleted DMEM (A) (Paper I, Figure 3A). In conditions, D-E in arginine-supplemented DMEM, there was no significant difference between the amount of endothelial NO produced by bradykinin-stimulated cells indicating that arginine uptake into the cells was saturating under these conditions (Paper I, Figure 3B).

Interestingly, arginine-supplemented DMEM with exogenous citrulline (F) caused a significant increase in bradykinin-stimulated endothelial NO production over that of stimulated endothelial NO production in arginine-sufficient DMEM (D-E) (Paper I, Figure 3B). Taken together, these data show that even in the presence of saturating levels of arginine, citrulline is a limiting factor and that regeneration of arginine from citrulline provides the substrate for eNOS necessary for endothelial NO production (Paper I and Appendix C).

These results indicated an efficient recycling of arginine from citrulline in endothelial cells. Therefore we decided to study protein-protein associations of the enzymes of the citrulline-NO cycle. Earlier it was shown that urea cycle intermediates channel substrates from one enzyme to the next for efficient reactions (Cheung et al. 1989). Therefore, we proposed that the citrulline-NO cycle enzymes might be colocalized to the same cellular compartment for efficient enzyme reactions. Endothelial cells contain specialized plasma membrane compartments, called caveolae, that are rich in cholesterol and sphingolipids and are known to contain many signaling molecules (e.g. 
eNOS) (Garcia-Cardena et al. 1997; Sase & Michel 1997). We performed two methods of endothelial caveolae membrane isolation (Smart et al. 1995; Song et al. 1996). Caveolae membranes were isolated by a detergent-free alkaline method and a detergent-free non-alkaline method of density separation. We collected fractions from each method and spotted these onto nitrocellulose membranes and probed with antibodies against eNOS, AS and AL along with the structural protein of caveolae, caveolin-1. Using the detergent-free non-alkaline method (Paper I, Figure 4) and the detergent-free alkaline method (Paper I, Figure 5), citrulline-NO cycle proteins co-fractionated in pooled fractions 4-5 or pooled fractions 3-4, respectively. Then we took the same fractions from the detergent-free alkaline method, separated the proteins by SDS-PAGE and used western blotting with antibodies against eNOS, AS and caveolin-1 to detect these citrulline-NO cycle proteins in the caveolae membrane fraction, pooled fractions 3-4 (Paper I, Figure 6). We also used an antibody against GM130, a golgi matrix protein and found that this protein co-fractionated with the pooled cytosolic fractions 5-10 indicating our fractionation protocol was successful. A protein assay confirmed that most protein was contained in the cytosolic fractions (Paper I, Figure 6). We concluded that the enzymes involved in the citrulline-NO cycle, eNOS, AS and AL, were contained in the same cell compartment and could represent an efficient arginine regeneration mechanism for endothelial NO production (Paper I and Appendix C).

It was previously shown by in vitro binding assays that both eNOS and caveolin-1 and eNOS and heat shock protein 90 (hsp90) directly associated with one another (Fontana et al. 2002; Garcia-Cardena et al. 1997; Ghosh et al. 1998; Gratton et al. 2000; Ju et al. 1997). Venema et al. also showed an interaction through hsp90 between eNOS and soluble guanylate cyclase when NO binds to the heme within the soluble guanylate cyclase (Venema et al. 2003). eNOS interacting protein (NOSIP) was shown to associate with eNOS promoting its translocation from the caveolae to other subcellular fractions (Dedio et al. 2001) and overexpression of NOSTRIN caused a decrease in eNOS activity due to eNOS translocation away from the plasma membrane (Oess et al. 2006; Zimmermann et al. 2002).
AS and AL are also regulated by the intracellular environment and differences in regulation vary among cell types. In the liver, AS and AL play important roles in the urea cycle to remove excess nitrogen. However, these enzymes are expressed in many cell types, including endothelial cells which do not produce urea. It is important to note that in hepatocytes, AS and AL are localized to the outer mitochondria membrane (Cohen 1996) while in the endothelium, AS and AL co-fractionate with the caveolae membrane fraction (Paper I). As mentioned previously, the significance of AS and AL expression in endothelial cells was not realized until the endothelial-derived relaxing factor was identified as NO and that the other product of the reaction catalyzed by eNOS, citrulline, could be recycled to arginine. Pendleton et al. demonstrated that the mRNA coding regions for AS isolated from liver and the endothelium are the same (Appendix B). However, there are two variant forms of AS mRNA containing extended 5’ untranslated regions (UTR) found only in the endothelium that may account for tissue-specific differences. These AS mRNA variants may cause endothelial AS to be localized to different subcellular fractions and may influence protein-protein interactions between AS and other citrulline-NO cycle components. AS, AL and eNOS contain transcriptional regulatory domains within their promoters that may cause differential expression depending on the stimuli received by the endothelium. For example, if arginine is replaced with citrulline in tissue culture using a human nasal septum squamous cell carcinoma line, there is increased transcription of the AS gene (Jackson et al. 1988a). Based on observations of citrulline-NO cycle protein co-fractionation and variant endothelial-specific AS mRNA 5’ UTRs in endothelial cells (Paper I and Appendix B and Appendix C), we decided to use in vitro interaction assays to further explore precise protein-protein interactions.

AS and AL proteins were bacterially-expressed as glutathione-S-transferase (GST) or hexahistidine-T7 tag (6xHis) fusion proteins in the Rosetta 2 E. coli strain. This particular E. coli strain improves eukaryotic protein yield by incorporating a plasmid in the Rosetta 2 to express seven rare prokaryotic tRNA. pGEX-5X vectors were used for GST protein expression and pET-28 vectors were used for 6xHis protein expression. The Studier method of autoinduction was used for recombinant protein expression (Studier
GST fusion proteins were purified using glutathione agarose for GST-AS and GST-AL (Paper II, Figures 1A, 1C). 6xHis fusion proteins were purified using Ni-NTA agarose (Paper II, Figures 1A, 1B). The in vitro interaction assay uses a fusion-tagged recombinant protein as bait and either in vitro translated protein, recombinant protein or an endothelial cell lysate as prey. The bait protein is bound to a solid support, typically agarose beads, and the prey protein is added and the mixture is incubated for several hours. Following extensive washing to remove non-specifically bound proteins, reducing sample buffer is added to the agarose beads. Bait and prey proteins are separated by SDS-PAGE and western blotting with an antibody against the prey protein is used to detect the protein binding partner. There is a direct protein-protein interaction if a protein band is visible in the samples containing bait and either in vitro translated protein or recombinant protein as prey. There is a protein-protein association if a protein band is visible in the samples containing bait and endothelial cell lysate as prey. Since the endothelial cell lysate contains many proteins, it cannot be assumed that the interaction is direct.

In vitro translated (ivt) AS was incubated with GST-AL, GST-AS or GST. Both GST-AL and GST-AS were able to pull down the ivt AS. This demonstrated a direct interaction between these proteins (Paper II, Figure 2). This is not surprising due to their involvement in the citrulline-NO cycle for arginine regeneration and since AS and AL both exist as homotetrameric proteins (O'Brien 1979; O'Brien et al. 1981). These data confirm and extend our co-fractionation studies where endothelial AS and AL co-fractionated with one another.

Next we looked for protein-protein associations in unstimulated or stimulated endothelial cells using bacterially expressed AS or AL. As shown previously by Hellermann et al. (Appendix A) and others (Venema et al. 2003), activation of proteins upstream of and involved in the citrulline-NO cycle can alter their associations. Unstimulated or bradykinin plus sodium orthovanadate-stimulated endothelial cell lysates were prepared. 6xHis-AL or 6xHis-AS fusion proteins were incubated with these unstimulated or stimulated endothelial cell lysates. An association between eNOS and 6xHis-AL or 6xHis-AS was detected with an antibody against eNOS (Paper II, Figure 3) and an association between caveolin-1 and 6xHis-AL or 6xHis-AS was detected with an
antibody against caveolin-1 (Paper II, Figure 4). There did not appear to be a significant
difference in the amount of eNOS or caveolin-1 that was pulled down by either 6xHis-AL
or 6xHis-AS from unstimulated or stimulated endothelial cell lysates. These data confirm
and extend our earlier observations of a co-fractionation of AS and AL with eNOS and
caveolin-1 from endothelial cell caveolae membranes.

While these data presented in Papers I and II are exciting and support our
hypothesis of a close functional association among components of the citrulline-NO
cycle, namely, AS, AL, eNOS and caveolin-1, further research on direct associations with
known or unknown protein partners is needed. For example, ivt or recombinant eNOS
could be mixed with AS or AL fusion proteins to determine whether these proteins have a
direct interaction. Additionally, mutations at sites of reversible phosphorylation and O-
glycosylation could be made to ivt AS, AL and eNOS. These mutated ivt proteins could
be mixed with GST or 6xHis fusion protein partners to determine the effects of these
modified residues on protein-protein interactions in the citrulline-NO cycle. Different
domains of the ivt proteins could be prepared to resolve the exact amino acids involved in
direct protein-protein interactions. Also, citrulline-NO cycle proteins with GST, 6xHis or
other fusion tags could be transfected into endothelial cells. The cells could then be left
unstimulated or stimulated to produce more NO. Following the treatments, lysates could
be prepared and using affinity chromatography to isolate the fusion tagged proteins and
interacting protein partners, potentially novel protein binding partners could be detected
using immunoblotting or mass spectrometry techniques. This is a more complex system,
but may give a better picture of protein-protein interactions involved in endothelial NO
generation.

Post-translational modifications of a protein can change protein-protein
interactions or protein subcellular localization leading to alternative cellular pathways.
For example, phosphorylation of bovine eNOS on serine 1179, serine 635, serine 617 and
tyrosine 83 enhances its activity (Fulton et al. 2005; Gallis et al. 1999; McCabe et al.
2000; Michell et al. 2002). Serine 116 phosphorylation decreases eNOS activity, while
phosphorylation on threonine 497 inhibits eNOS activity (Corson et al. 1996; Fleming et
al. 2001; Kou et al. 2002; Schulz et al. 2005). Threonine 497 dephosphorylation causes
eNOS to produce superoxide and NO and that the balance of production of NO over superoxide can be modulated by eNOS accessory proteins or phosphorylation (Lin et al. 2003). Church and Fulton reported on differences seen in bovine eNOS activity between wild-type eNOS and mutated eNOS that remains cytosolic (Church et al. 2006). The mutated eNOS was unable to undergo acylation and remained in the cytosol, and thus never translocated to the plasma membrane or Golgi. They reported that the differences seen in activity were due to differences in the phosphorylation state of serines 617, 635, 1179. This caused a decrease in the amount of calcium-calmodulin binding to eNOS causing less dimerization of the enzyme and reduced enzyme activity. These changes in the acylation state of eNOS could have implications on protein-protein interactions between eNOS and known binding partners especially if the enzyme is unable to target a particular subcellular fraction.

Bradykinin, a biologically active polypeptide from blood globulin (Bae et al. 2003) and sodium orthovanadate, a tyrosine phosphatase inhibitor (Appendix A), are effectors which increase endothelial NO production. Hellermann et al. determined that BAEC stimulated with sodium orthovanadate plus either bradykinin or calcium ionophore produced more endothelial NO than the sum of endothelial NO produced by either sodium orthovanadate plus bradykinin or sodium orthovanadate plus calcium ionophore. Sodium orthovanadate did not have a direct effect on cellular lysate eNOS activity in vitro. Hellermann et al. also performed endothelial cell lysate fractionation and showed that sodium orthovanadate did not change the subcellular localization (caveolae versus cytosolic) of eNOS. However, sodium orthovanadate plus bradykinin endothelial cell treatment resulted in an increase in tyrosine phosphorylation of three proteins of apparent 75, 105 and 125 kDa. They concluded that the increase in NO production seen with sodium orthovanadate plus bradykinin treatment was due to a change in the association of proteins with eNOS, perhaps enhancing its activity (Appendix A).

The concept of arginine regeneration from citrulline for endothelial NO production has been explored in great detail. For example, researchers have sought to determine the cause of the “arginine paradox” (Hardy & May 2002). Earlier it was demonstrated that endothelial cells had the capacity to recycle citrulline to arginine in
order to have arginine available for NO synthesis (Hecker et al. 1990b; Wu & Morris 1998; Wu & Meininger 1993) (Paper I). They concluded that citrulline was recycled to arginine by AS and AL. This recycling pathway would allow for arginine availability to eNOS during periods of sustained NO production. Van Geldre et al. showed that in muscle layer nerve fibers and in neurons in the myenteric plexus in the rat gastric fundus, AS and AL co-localized with nNOS. Using smooth muscle strips, they also showed that administration of arginine completely, and citrulline partially, prevented L-NAME NOS-inhibition of muscle relaxation (Van Geldre et al. 2002). Mohacsi et al. demonstrated that arginine availability was not limiting for recombinant eNOS activity so adenovirus mediated eNOS transfer into rabbit aorta was performed which improved vascular relaxation with or without arginine administration (Mohacsi et al. 1999). Simon et al. demonstrated that there are different intracellular compartments of arginine depending on cell type and eventual fate (Simon et al. 2003). Tsikas et al. noted that only 0.1% of administered arginine enters the L-citrulline-NO cycle (Tsikas et al. 2006) suggesting that endothelial cells must have a functioning arginine regeneration system in place for endothelial NO production. McDonald et al. demonstrated using confocal microscopy in porcine aortic endothelial cells (PAEC) that caveolin-1, the structural protein of caveolae co-localized with the cationic amino acid transporter 1, system y+ (CAT1) and that eNOS and CAT1 co-localized in the PAEC caveolae (McDonald et al. 1997). They concluded that the intracellular arginine was inaccessible to eNOS and that eNOS was co-localized with the arginine transporter, CAT1, at the plasma membrane surface to allow for NO production from arginine transported into the cell. More recently, Li et al. established that eNOS and CAT1 directly interact, but that this interaction increases NO production in an arginine transport-independent manner (Li et al. 2005).

Therefore we proposed to directly measure the production of both NO and citrulline in unstimulated (basal) and sodium orthovanadate plus bradykinin stimulated (stimulated) endothelial cells to determine the degree of recycling of citrulline to arginine. $^{14}$C-Arginine was added to the endothelial cells and after 90 minutes, the reaction was stopped and both NO from the tissue culture medium and citrulline from the cells were measured in the samples. To prevent arginine depletion by arginase, all cells
were treated with \((S)-(2\text{-Boronoethyl})\)-L-cysteine hydrochloride (BEC-HCl), an inhibitor of arginases I and II (Berkowitz et al. 2003). Some cells were also treated with L-NAME, an eNOS inhibitor, to determine eNOS-specific NO and citrulline generation. We determined that under both basal and stimulated conditions, the ratios of NO (measured as nitrite, an end-product) formed to citrulline were 8:1 (Paper III, Table I and Figure 2). The apparent amount of NO compared to citrulline produced in the basal or stimulated state was significantly greater, indicating that recycling of citrulline to arginine and conversion of arginine to NO is very efficient under all conditions. We also showed that due to arginine recycling, eNOS activity and the degree of stimulation can be substantially underestimated if measurements are made based on citrulline production alone.

Inhibiting AS activity in endothelial cells should prevent the recycling of citrulline to arginine. To determine the direct effects of stimulation on the citrulline-NO cycle, we used \(\alpha\)-methyl-DL aspartic acid (MDLA), an AS inhibitor (Shen et al. 2005). Sodium orthovanade plus bradykinin-stimulated endothelial cells were incubated in increasing concentrations of MDLA and again, simultaneous measurement of NO and citrulline was performed. Although NO production was inhibited by 80% at the highest concentration of MDLA, the ratio of NO to citrulline was 7:1, indicating a substantial degree of coupling where most of the citrulline produced was recycled to arginine for NO production (Paper III, Figure 3).

Lastly, to show that recycling of arginine occurs within a specific intracellular location, we deprived endothelial cells of arginine for 48 hours. Arginine-sufficient (cultured in the presence of 340 \(\mu\)M arginine) or arginine-deprived cells (cultured in the presence of \(~10\ \mu\)M arginine) were either left in the basal state or stimulated, and NO production was determined (Paper III, Figure 4). If endothelial NO production was dependent on bulk intracellular arginine, which ranges between 0.1-0.8 mM, then we would have expected to see a dramatic decrease in the amount of NO produced in the arginine deprived versus arginine sufficient cells. There was only a modest decrease in NO production by arginine-deprived cells, relative to the extent of arginine depletion,
consistent with our hypothesis that the primary source of arginine directed to NO production is provided by the citrulline-NO cycle.

Endothelial cell function and citrulline-NO cycle proteins are also affected by the extracellular environment. Increases in NO production are rapid and are caused by reversible phosphorylation, O-glycosylation, protein-protein associations, cellular translocation and substrate/cofactor availability of citrulline-NO cycle proteins. A specific protein modification, O-glycosylation, leads to rapid changes in protein function. Researchers determined that O-glycosylation of the transcription factor, Sp1, causes its translocation into the nucleus where it binds to response elements in the AS promoter region leading to increased gene transcription (Anderson & Freytag 1991; Brasse-Lagnel et al. 2003). Glucose, glutamine and glucosamine enter the hexosamine biosynthetic pathway increasing UDP-GlcNAc concentrations, so it is possible that cell culture conditions of high glucose, glutamine or glucosamine or a combination can change endothelial cell nitric oxide production or O-linked β-N-acetylg glucosamine (O-GlcNAc, Appendix D) protein modifications.

Incubation of endothelial cells with glucosamine causes increased O-GlcNAc modifications on plasminogen activator inhibitor 1 (PAI-1) (Goldberg et al. 2002). As previously mentioned, Sp1 is O-GlcNAc modified and it binds to transcription sites in the PAI-1 promoter causing increased translation of PAI-1 (Du et al. 2000). Glucosamine increased O-GlcNAc modifications on eNOS protein near the PKB phosphorylation site (Du et al. 2001). This increased activity of PAI-1 along with decreased eNOS activity due to O-GlcNAc modifications may cause PAI-1 overexpression leading to pathological atherosclerosis (Federici et al. 2002; Swiatkowska et al. 2000). Werstuck et al. recently demonstrated that high glucosamine and glucose concentrations caused protein misfolding in the endoplasmic reticulum, leading to ER stress, and that this ER stress was correlated with atherogenic lesion development in apoE-deficient mice (Werstuck et al. 2006). Glucosamine induces insulin resistance at lower concentrations than glucose (Vosseller et al. 2002). Incubation of heart and skeletal muscle with high concentrations of glucosamine increases glucosamine-6-phosphate concentrations resulting in depleted intracellular ATP levels within the cell (Buse 2006). Buse et al. also saw changes in gene
expression in 3T3-L1 adipocytes incubated with 2.5 mM glucosamine and 0.6 nM insulin and either low glucose (5 mM) or high glucose (25 mM). Endothelial cells experience similar changes in gene expression where these changes may only be seen after exposure to glucosamine (Du et al. 2000; Goldberg et al. 2002).

We designed experiments to determine changes in endothelial NO production and citrulline-NO cycle proteins in BAEC, resulting from either high glucose, glutamine or glucosamine treatment. The amount of NO produced by BAEC after 20-24 hours in the presence of 5 mM glucose and either low glutamine (0.2 mM) or high glutamine (2 mM) was significantly greater with the low glutamine treatment. The amount of NO produced by BAEC after 20-24 hours in the presence of 20 mM glucose and either low glutamine (0.2 mM) or high glutamine (2 mM) was greater with the low glutamine treatment. To prevent conversion of glutamine to UDP-GlcNAc, the substrate for O-GlcNAc transferase, BAEC were treated with 50 µM 6-diazo-5-oxo-L-norleucine (DON, Appendix D), a glutamine amidotransferase inhibitor of the rate limiting-step of the hexosamine biosynthetic pathway. Using the DON inhibitor, the amount of NO produced by BAEC after 20-24 hours in the presence of 2 mM glutamine and either 5 mM or 20 mM glucose was increased as compared to cells not treated with the inhibitor (Paper IV, Table 1). These data were in agreement with Wu et al. who showed that using a GFAT inhibitor (DON) to prevent production of UDP-GlcNAc demonstrated the involvement of glutamine in the hexosamine biosynthetic pathway (Wu et al. 2001a). These data were also in agreement with Du et al. who showed that O-GlcNAc modifications decrease eNOS activity leading to lowered endothelial NO production (Du et al. 2001).

Next we measured NO production in basal (unstimulated) or bradykinin-stimulated endothelial cells treated with glucosamine for 20-26 hours. Under basal conditions there was a significant dose dependent increase in NO production at 2.1, 10.5 and 15 mM glucosamine (Paper IV, Figure 2). However, under bradykinin-stimulated conditions, there was a dose dependent decrease in NO production at 2 and 8 mM glucosamine (Paper IV, Figure 3). The dose dependent effects of glucosamine observed under basal conditions were completely opposite to the results seen by Wu et al. (Wu et al. 2001a). The differences seen between the two experiments may be due to the source
of the endothelial cells and the cells were maintained in different concentrations of glucose. Our endothelial cells are typically maintained in 5 mM glucose (physiological), while Wu et al. maintained their endothelial cells in 20 mM glucose.

Under conditions of high extracellular glucose, glutamine or glucosamine, alone or in combination, O-GlcNAc substitution of Sp1 increases leading to increased AS promoter binding and increased AS transcription (Anderson & Freytag 1991; Brasse-Lagnel et al. 2003). Nandi et al. showed that AS can be O-GlcNAc modified, which we speculate may improve/enhance its enzyme activity or its ability to associate with proteins involved in endothelial NO generation (Nandi et al. 2006). Either the increased activity or quantity of O-GlcNAc modified AS, induced by high extracellular concentrations of glucose, glutamine or glucosamine, may be able to overcome the inhibition caused by O-GlcNAc modification of eNOS under basal conditions pushing the citrulline-NO cycle to produce more NO. However, under stimulated conditions, if eNOS is O-GlcNAc modified and has decreased activity, even endothelial cells having increased AS activity due to O-GlcNAc modification or quantity induced by high extracellular concentrations of glucose, glutamine or glucosamine, may be unable to generate NO at the same rate as cells not treated with glucosamine.

We then looked at the levels of eNOS and AS using protein specific antibodies and also O-GlcNAc modification of proteins using an antibody to detect O-GlcNAc protein modifications in cell lysates from bradykinin-stimulated endothelial cells in the absence and presence (2 mM and 8 mM) of glucosamine. We found increases in total eNOS and AS proteins upon incubation with 2 mM and 8 mM glucosamine for 26 hours (Paper IV, Figure 4). Glucosamine addition caused a slight increase in total cellular O-GlcNAc modified proteins in bradykinin-stimulated endothelial cell lysates (Paper IV, Figure 5). AS protein levels increased with glucosamine addition as expected. In the presence of high glutamine concentrations, Brasse-Lagnel et al. showed increased UDP-GlcNAc levels, the substrate for O-GlcNAc transferase, leading to increased O-GlcNAc modified Sp1, which translocates to the nucleus and increases AS gene transcription in Caco-2 cells (Brasse-Lagnel et al. 2003). Our results which showed an increase in total endothelial cellular O-GlcNAc protein modifications upon incubation with glucosamine.
are in agreement with Du et al. who showed an increase in total cellular O-GlcNAc modified endothelial cell proteins upon incubation with high glucose (Du et al. 2001). Du et al. did not see a change in total cellular eNOS protein levels upon incubation with high glucose, although they showed an increase in O-GlcNAc modified eNOS protein along with a decrease in phosphorylated (serine 1177) eNOS protein. Our results showed an increase in eNOS protein levels upon incubation with 2 and 8 mM glucosamine. The differences between the two experiments may be due to the source of the eNOS protein. Du et al. transfected a plasmid encoding myc-tagged human eNOS into BAEC and then detected total eNOS protein with antibodies against myc (human eNOS) or eNOS (human and bovine). In our experiments, total cellular bovine eNOS protein was detected using an antibody against eNOS. Although we saw a decrease in eNOS activity (based on NO production) along with an increase in total cellular eNOS protein, it is possible that this additional eNOS protein was not enzymatically active and therefore would not contribute to endothelial NO production.

While these data presented in Paper IV are intriguing, additional research to determine the effects of glucose, glutamine or glucosamine alone or in combination on expression and activity of citrulline-NO cycle enzymes is needed. For example, using immunoprecipitation with antibodies or lectins (which bind to the O-GlcNAc moiety) it would be important to measure cellular levels of O-GlcNAc modified eNOS and AS, in the presence of varying concentrations of glucose, glutamine or glucosamine alone or in combination and compare the amount of modified proteins found in the presence or absence of the inhibitors DON or O-(2-acetamido-2-deoxy-D-glucopyranosylidene)-amino-N-phenylcarbamate (PUGNAc, Appendix D). DON inhibits the rate limiting step of the hexosamine biosynthetic pathway, preventing the production of UDP-GlcNAc (moiety for O-GlcNAc modifications) from glucose and glutamine. PUGNAc is an O-GlcNAcase inhibitor and specifically prevents the removal of the O-GlcNAc protein modification. By comparing results from western blot data plus and minus inhibitors, the pathways involved in AS and eNOS protein modifications may be further elucidated. Also, nuclear extracts from cell experiments similar to those outlined above should be performed. Electrophoretic mobility shift assays could be performed to look for
differences in binding to specific promoter regions known to contain binding sites for Sp1 (eNOS, AS and AL) or NF-Y (AL) in cells treated with glucose, glutamine or glucosamine alone or in combination, plus and minus the inhibitors DON and PUGNAc.

In summary we showed that citrulline and arginine increased endothelial NO production (Paper I and Appendix C). We also showed a co-fractionation of the citrulline-NO cycle proteins, eNOS, AS and AL with the endothelial caveolae membrane fraction (Paper I and Appendix C). Using \textit{in vitro} interaction assays, we showed a direct association between AS-AL and an interaction between AS-eNOS, AL-eNOS and AL-caveolin-1 providing further proof for protein-protein interactions between citrulline-NO cycle proteins (Paper II). Pendleton et al. (Appendix B) provided evidence for a difference in endothelial and liver AS targeting, while Hellermann et al. (Appendix A) provided evidence for a change in eNOS protein-protein interactions upon endothelial cell stimulation with sodium orthovandate plus bradykinin. We then showed evidence for a tight coupling of the reactions of the citrulline-NO cycle, to ensure citrulline recycling to arginine for endothelial NO production by eNOS (Paper III). Finally we provided evidence that the endothelial extracellular environment plays a crucial role in the function of citrulline-NO cycle proteins (Paper IV). We and others have shown that NO production is necessary to maintain cell viability (Goodwin et al. 2004), that a close association of proteins of the citrulline-NO cycle provides a more efficient method to produce endothelial NO by regenerating its precursor arginine from a specific intracellular source and location (Papers I-III) and that the endothelial extracellular environment can change protein function (Paper IV). It is hoped that the contribution of the studies from our laboratory will lead to increased understanding of the expression, localization and regulation of citrulline-NO cycle enzymes and their role in endothelial function and NO production.
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Appendices
Appendix A

Stimulation of Receptor-Mediated Nitric Oxide Production by Vanadate
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Abstract

Nitric oxide (NO) production by endothelial cells in response to bradykinin (Bk) treatment was markedly and synergistically enhanced by cotreatment with sodium orthovanadate (vanadate), a phosphotyrosine phosphatase inhibitor. This enhancement was blocked by tyrosine kinase inhibitors. Calcium ionophore – (A23187) activated production of NO was also enhanced by cotreatment with vanadate. No significant changes were found in total endothelial NO synthase (eNOS) protein or in eNOS distribution between membrane (caveolae) and cytosolic fractions in response to the various treatments. Vanadate had no direct effect on eNOS activity, and lysates prepared from cells treated with vanadate showed little change in specific activity of eNOS. Western blots of immunoprecipitated eNOS showed the presence of a major tyrosine-phosphorylated protein band at a mass corresponding to ≈125 kDa and 2 minor bands corresponding to ≈105 and 75 kDa after treatment with vanadate/Bk. No tyrosine phosphorylation of eNOS after treatment with vanadate/Bk was observed. Geldanamycin, an inhibitor of heat shock protein 90, also inhibited the enhancement of NO production by vanadate/Bk or vanadate/A23187, and there was an increase in the amount of heat shock protein 90 that coimmunoprecipitated with eNOS after treatment with vanadate/Bk. These results show that there is a clear link between tyrosine phosphorylation and stimulation of eNO production, which does not appear to involve direct modification of eNOS, changes in eNOS levels, or compartmentation, but rather appears to be due to changes in proteins associating with eNOS, thereby enhancing the state of activation of eNOS.

Key Words: nitric oxide, endothelial, vanadate, bradykinin, tyrosine phosphorylation
Endothelial nitric oxide synthase (eNOS) plays a key role in vasoregulation through the highly regulated production of eNO, which diffuses to the smooth muscle layer, causing relaxation. Several studies have suggested a linkage between phosphorylation/dephosphorylation events and eNO production. An early study by Michel et al (Michel et al. 1993) demonstrated that short-term incubation of endothelial cells with bradykinin (Bk) or calcium ionophore A23187 led to serine phosphorylation of eNOS, which appeared to be associated with intracellular translocation of eNOS (Prabhakar et al. 1998). More recent studies have demonstrated that Akt (protein kinase B) activates eNOS by phosphorylation of a specific serine residue (Dimmeler et al. 1999; Fulton et al. 1999; Michell et al. 1999). The involvement of tyrosine phosphorylation in eNOS regulation has also been studied. Exposure of endothelial cells to vanadate resulted in a low level of tyrosine phosphorylation, compared with a much greater level of serine phosphorylation (Garcia-Cardena et al. 1996). The in vitro activity measured on immunoprecipitates of eNOS suggested that tyrosine phosphorylation reduced the activity of eNOS by about one half (Garcia-Cardena et al. 1996). Fleming et al (Fleming et al. 1998) found a low level of basal tyrosine phosphorylation of eNOS, which was lost after treatment of endothelial cells with a phospho-tyrosine phosphatase inhibitor (phenylarsine oxide). Venema et al (Venema et al. 1996) observed neither basal nor Bk-mediated stimulation of tyrosine phosphorylation of eNOS but did find that Bk activation of eNOS was accompanied by tyrosine phosphorylation of an eNOS-associated protein (ENAP-1). Activation of eNOS by flow-mediated shear stress appears to involve tyrosine phosphorylation, the activation being reduced by tyrosine kinase inhibitors and stimulated by inhibitors of phosphotyrosine phosphatases (Corson et al. 1996; Fleming et al. 1997; Fleming et al. 1998; Fleming et al. 1996). Corson et al (Corson et al. 1996) proposed that shear stress–mediated eNOS activity might be regulated by phosphorylation of the enzyme. They found an increase in serine/threonine phosphorylation, but not tyrosine phosphorylation, of eNOS after application of shear stress. Thus, the precise role of tyrosine phosphorylation in the regulation of eNOS activity is still uncertain.
In the present study, we examined the effect of phosphotyrosine phosphatase inhibition by sodium orthovanadate (vanadate) on eNO production and found an unexpectedly large and sustained increase in the amount of eNO produced after Bk-or calcium ionophore–mediated activation of eNOS. Although eNOS does not appear to be directly tyrosine-phosphorylated under these conditions, changes in the level of certain eNOS-associated phosphotyrosine-containing and other proteins suggest a role for tyrosine phosphorylation/ dephosphorylation in the physiological regulation of eNOS activity.
Methods

**Cell Culture** – Endothelial cells used in this study were isolated from bovine aortas (BAECs) according to a standard method (Gimbrone 1976) and verified as endothelial by positive immunostaining for von Willebrand factor, negative staining for muscle actin, and morphological examination by electron microscopy (Jaffe 1984). Cells were routinely seeded in 12-well culture plates (Corning/Costar) at dilutions of 1:3 to 1:5 and grown to confluence in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 U penicillin, 100 µg streptomycin, 0.25 µg amphotericin B, and 50 µg gentamycin per milliliter at 37°C in an atmosphere of 95% air/5% CO₂.

**Treatment Conditions** – Cells were used 2 to 3 days after reaching confluence at a density of 1.5 to 1.7x10⁵ cells/cm² and >98% viability by the trypan blue dye-exclusion test. Cell monolayers in 12-well cluster dishes were rinsed 3 times with standard PBS at 37°C, followed by the addition of 0.7 mL of Dulbecco’s modified Eagle’s medium containing antibiotics but no serum to each well. The NOS inhibitor N⁶-nitro-L-arginine methyl ester (L-NAME) was added, where indicated, to a final concentration of 1 mmol/L and incubated for 30 minutes at 37°C before addition of the test compounds. Phosphatase or kinase inhibitors were added 5 minutes before eNOS activation with Bk, A23187, vanadate, vanadate/Bk, or vanadate/A23187. Incubations were carried out at 37°C and test compounds were present throughout the incubation period. Solutions of sodium orthovanadate at a concentration of 5 mmol/L were prepared by heating at 95°C for 10 minutes, and actual concentrations of orthovanadate were determined by spectrophotometric measurement at 260 nm by using a millimolar extinction coefficient of 3.55 (Gordon 1991).

**Assay for NO as Nitrite in Culture Medium** – The method for measuring nitrite, a stable reaction product of NO, involves the conversion of 2,3-diaminonaphthalene to the highly fluorescent 1(H)-naphthotriazole through specific reaction with nitrite under acidic conditions (Misko et al. 1993). Cell counts were performed on a sampling of wells by using a microscope with an eyepiece micrometer, and the average was used in converting the readings to a per-10⁶-cell basis. Fluorescence intensity was determined at room temperature with a Jasco FP-770 spectrofluorometer.
**Preparation of Caveolin-Enriched Membrane Fractions** – The method of Song et al (Song et al. 1996) was used to prepare caveolae from BAECs. All steps were carried out at 4°C. In brief, confluent cell monolayers were treated with various agents, washed with PBS, and suspended in 500 mmol/L Na₂CO₃, pH 11. The alkaline cell suspension was lysed with a Dounce homogenizer followed by further homogenization with a Polytron tissue homogenizer and brief sonication, as described by Song et al (Song et al. 1996). One milliliter of each lysate was mixed with an equal volume of 90% sucrose, transferred to the bottom of a centrifuge tube, and overlaid with a step gradient consisting of 1.0 mL of 35% sucrose, 0.5 mL of 25% sucrose, 0.5 mL of 15% sucrose, and 0.5 mL of 5% sucrose, all with the same buffer composition as the 45% sucrose sample solution. Tubes were centrifuged in an SW60 Ti rotor at 40 000 rpm for 17 hours. Fractions of 0.4 mL were collected from the top of the gradient. The caveolar peak corresponded to fraction 3.

**Preparation and Processing of Cell Lysates** – After the indicated incubation period, cells were rinsed 3 times with cold PBS and stored at -80°C until processed. Lysates were prepared by scraping cells from each well into 250 µL of cell-disruption buffer containing 20 mmol/L HEPES (pH 7.5); 0.1 mol/L NaCl; 5 µg/mL each of pepstatin A, leupeptin, and trypsin inhibitor; 0.5 mmol/L PMSF; 0.1 mmol/L DTT; 1 mmol/L sodium orthovanadate; and 10 mmol/L KF. Three rounds of freeze/thaw or three 6-second bursts of sonication with a microprobe at a 25-W output were sufficient to lyse >90% of the cells, as judged microscopically. Cell lysates were used directly, separated into caveolar fractions (see above), or separated into membrane and cytosolic fractions by 15 minutes of centrifugation at 100 000g in a TL-100 ultracentrifuge with a fixed-angle rotor. Protein determinations were done using the bicinchoninic acid protein assay kit (Pierce) with bovine serum albumin as the standard.

**Immunodetection of Proteins and Immunoprecipitation** – For determination of eNOS protein levels, aliquots of cell lysates or fractions were either directly adsorbed to nitrocellulose membranes with a dot-blot apparatus (Bio-Rad) or separated by SDS–polyacryl-amide gel electrophoresis (SDS-PAGE) and electroblotted onto nitrocellulose membranes. For immunoprecipitations, cell lysates (~100 µg protein) were incubated
with a specific antibody (≈1 µg) for 1 hour at 4°C, followed by 1 hour at 4°C with protein A/G Sepharose (Sigma). Precipitated proteins were solubilized with SDS-PAGE sample buffer and electrophoretically separated. Blots were blocked in 1% gelatin or 5% nonfat dry milk in 10 mmol/L Tris·HCl (pH 7.5), 0.1 mol/L NaCl, and 0.2% Tween 20 and then incubated, where indicated, with 1:1000 dilutions of monoclonal antibodies specific for eNOS (Transduction Labs), caveolin-1 (Transduction Labs), protein tyrosine phosphate (clone PY20, Zymed Labs), or heat shock protein 90 (hsp90; StressGen Labs). Horseradish peroxidase–conjugated IgG (Transduction Labs) was used to detect the respective primary monoclonal antibody by means of a horseradish peroxidase–activated chemiluminescent substrate (enhanced chemiluminescence, Amersham). Densitometric measurements on scanned images were completed by using ImageQuaNT software (Molecular Dynamics).

**In Vitro Assay for eNOS Activity** – The possible direct effect of sodium orthovanadate on eNOS activity in lysates of endothelial cells was tested by using an eNOS assay based on the conversion of [³H]L-arginine to [³H]citrulline (Bredt et al. 1990). Assay mixtures contained 25 mmol/L HEPES (pH 7.4), 3 µmol/L tetrahydrobiopterin, 1 µmol/L FAD, 1 µmol/L flavin mononucleotide, 1 mmol/L NADPH, 0.6 mmol/L CaCl₂, 0.1 µmol/L calmodulin, and 20 µCi/mL [³H]L-arginine (60 Ci/mmol). Assays were run for 30 and 60 minutes in the presence or absence of 1 mmol/L sodium orthovanadate at 37°C, and activity was compared with background levels in the absence of calcium or in the presence of the NOS inhibitor L-NAME. Lysates from cells treated with Bk and/or vanadate were also assayed for eNOS activity to determine whether the treatments resulted in any change in specific activity of eNOS.

**Statistical Analysis** – Data are expressed as mean ± SEM. At least triplicate determinations were performed for quantitative analyses. Student’s t test was used for evaluating significance.
Results

Vanadate-Stimulated eNO Production – Endothelial cells were incubated with the protein phosphotyrosine phosphatase inhibitor (Gordon 1991) sodium orthovanadate, and the rate of eNO production was measured during continuous exposure to either Bk or calcium ionophore A23187. A small, continuous basal level of eNO was produced in the absence of vanadate, Bk, or A23187. Bk stimulated eNO production to a level about double that of the untreated controls (Figure 1A), whereas A23187, which acts in a receptor-independent manner to directly raise the intracellular calcium concentration, caused a 4-to 5-fold increase in NO production (Figure 1B). Vanadate alone stimulated the formation of eNO in a time-dependent manner even in the absence of added Bk or A23187. When Bk or A23187 was added to endothelial cells in the presence of vanadate, however, there was a dramatic and sustained enhancement of eNO formation to levels 10 to 20 times that of controls (Figure 1). The amount of eNO generated by treatment with a combination of vanadate and either Bk or A23187 was >2-fold greater than the sum of eNO generated by treatment with the individual components, indicating a synergistic effect of vanadate and Bk or A23187 (Figure 1, summation curves).

We also examined the effect of phosphotyrosine phosphatase inhibition by vanadate over a range of Bk or A23187 concentrations. When cells were incubated with Bk (Figure 2A), the enhancing effects of 50 µmol/L vanadate were most apparent at the lower concentrations and reached a plateau as Bk activation peaked at ≈10 µmol/L. Incubation of endothelial cells with A23187 led to a rapid and sustained rise in eNOS activity due to calcium influx, and vanadate further stimulated eNO production. This enhancement was most striking at A23187 concentrations <5 µmol/L, but enhancement of eNO production by vanadate occurred even under conditions of apparent calcium saturation (Figure 2B). Incubation of cells in medium lacking calcium or containing EGTA completely abolished the sustained generation of eNO, indicating the necessity of a continuous influx of calcium from outside the cells for extended eNOS activity. Likewise, removal of L-arginine from the medium prevented the sustained generation of eNO (data not shown).
Effects of Protein Kinase and Other Inhibitors on Vanadate-Stimulated NO Production – The specific involvement of tyrosine phosphorylation in the enhancement of eNO production is shown in Figure 3. Cells were pretreated with the protein tyrosine kinase inhibitors genistein (Akiyama et al. 1987) or tyrphostin ST638 (Gazit et al. 1991) before activation with either Bk (Figure 3A) or A23187 (Figure 3B), and the effects of vanadate were abrogated. Phenylarsine oxide, which inhibits a class of phosphotyrosine phosphatases that contain vicinal sulfhydryls at the active site (Kussmann et al. 1995), in contrast to vanadate, which mimics the transition state of phosphotyrosine phosphatase–catalyzed reactions (Zhang et al. 1997), did not cause enhancement of eNO generation (Figure 3A), indicating that the class of phosphotyrosine phosphatases inhibited by phenylarsine oxide was not involved in the regulation of eNO production under these conditions. Pretreatment of endothelial cells with the NOS inhibitor L-NAME (Moncada et al. 1989) blocked eNO production, whereas co-treatment with dexamethasone, which blocks induction of the inducible isoform of NOS (Radomski et al. 1990), had little effect (Figure 3B). Treatment with the protein synthesis inhibitor cycloheximide did not alter the vanadate response over the first 2 hours, suggesting that the enhancement most likely involved a constitutive protein (data not shown). Okadaic acid, a potent inhibitor of the phosphoserine/phosphothreonine phosphatases pp1 and pp2a (Cohen et al. 1989), reduced the vanadate enhancement of eNO production in Bk-treated cells (Figure 3A). When endothelial cells were activated by A23187 in the presence of okadaic acid, however, there was no effect on the vanadate response, indicating that involvement of a putative serine kinase was specific for the Bk-mediated signaling pathway (Figure 3B). The same basic experiments were repeated with primary cultures of BAECs and human umbilical vein endothelial cells, with essentially the same results (data not shown).

Effect of Vanadate on eNOS Compartmentation and Expression – To determine whether vanadate affects the intracellular location of eNOS, the relative levels of eNOS protein were measured in cell lysates and subcellular fractions by immunoblotting and densitometry. After centrifugation of endothelial cell lysates at 100,000g, ≈95% of total eNOS protein was associated with the particulate fraction, and vanadate treatment, in the presence or absence of Bk, caused little or no change in this distribution. Also, there was
no significant difference in total eNOS protein under the various treatment conditions compared with controls. Treatment of endothelial cells with Bk, vanadate, or vanadate/Bk had no significant effect on the subcellular distribution of eNOS, as indicated by isopycnic centrifugation of cell lysates through a sucrose gradient under conditions designed to separate caveolae from denser membrane fractions (Song et al. 1996). Most of the eNOS protein was associated with the caveolar peak fraction (fraction 3) under all treatment conditions (Figure 4). This fraction corresponded to the interface between 15% and 25% sucrose. Noncaveolar membrane fractions were distributed in denser regions of the gradient (fractions 4 to 8) (Song et al. 1996). The presence of eNOS and caveolin-1 in the peak fractions shown in Figure 4 was confirmed by Western blotting (data not shown). Because the samples were loaded from the bottom of the gradients, cytosolic and cytoskeletal proteins would remain in the 45% sucrose sample zone (fractions 8 to 10).

**Effect of Vanadate on eNOS Activity Measured In Vitro** – To determine whether vanadate affected eNOS directly, lysates of untreated endothelial cells were assayed for eNOS activity in the presence or absence of vanadate. No effect of vanadate, at a concentration of 1 mmol/L, was observed (data not shown), indicating that vanadate had no direct effect on eNOS. Lysates prepared from cells that had been incubated with vanadate or vanadate/Bk for 4 hours showed a slight increase in eNOS activity compared with controls (31.2±2.4 versus 25.3±2.0 pmol citrulline produced per minute per milligram protein), but this possible increase in eNOS activity measured in vitro does not account for the striking increase in NO production by endothelial cells after treatment with vanadate/Bk.

**Vanadate-Induced Changes in Tyrosine-Phosphorylated Proteins** – The state of tyrosine phosphorylation of eNOS and other protein components after the various treatment conditions was analyzed by Western blotting, after immunoprecipitation with anti-eNOS, by using an antibody probe to protein tyrosine phosphate. A tyrosine-phosphorylated protein with an apparent molecular mass of ≈125 kDa, along with lesser amounts of tyrosine-phosphorylated proteins with apparent molecular masses of ≈105 and 75 kDa, appeared to be specifically associated with eNOS after vanadate/Bk treatment, as indicated by coimmunoprecipitation with anti-eNOS (Figure 5). The
Appendix A (Continued)

apparent molecular mass of eNOS run under identical conditions was ≈140 kDa. Little or no tyrosine phosphorylation of eNOS was observed under these conditions.

**Possible Role of hsp90 on Vanadate Stimulation of eNO Production** – hsp90 is a molecular “chaperone” with a molecular mass of ≈90 kDa that functions, in part, to facilitate the folding of certain signal-transducing proteins (Buchner 1999; Caplan 1999). hsp90 has been shown to associate with and stimulate eNOS activity (Garcia-Cardena et al. 1998). Geldanamycin, an inhibitor of hsp90 (Buchner 1999; Caplan 1999), inhibited the vanadate/Bk and vanadate/A23187 stimulation of eNO production with an apparent IC50 of ≈6 µmol/L (data not shown). Direct involvement of hsp90 in the enhancement of eNOS activity under these conditions was further supported by the finding that an increased amount of hsp90 coimmunoprecipitated with eNOS after vanadate/Bk treatment (Figure 6).
Discussion

Early studies with purified NOS showed that the enzyme could be a substrate for a number of protein kinases (Nakane et al. 1991). Although the bulk of in vivo eNOS phosphorylation occurs on serine/threonine, there have been reports of tyrosine phosphorylation of eNOS itself (Fleming et al. 1998; Garcia-Cardena et al. 1996). Garcia-Cardeña et al (Garcia-Cardena et al. 1996) found that treating endothelial cells with high levels (1 mmol/L) of vanadate caused an increase in basal tyrosine phosphorylation of eNOS and reduced eNOS activity by ≈50% in an in vitro immunoprecipitation-complex assay. Our results, in contrast, indicate that lower levels (50 µmol/L) of vanadate enhanced the Bk-and calcium ionophore–mediated production of eNO. This enhancement was apparently indirect, because there was no change in eNOS activity measured in vitro and no tyrosine phosphorylation of eNOS was observed. These apparently contradictory results may be due to different experimental conditions. We used a significantly lower concentration of vanadate (50 µmol/L versus 1 mmol/L) and focused on receptor-mediated production of eNO. It is unlikely that vanadate at the low concentration used in this study (50 µmol/L) could cause such an enhancement of eNOS activity through a nonspecific cellular effect. Venema et al (Venema et al. 1996) also reported that binding of Bk to endothelial cells did not elicit tyrosine phosphorylation of eNOS, but rather of a 90-kDa ENAP-1, which appeared to lead to the translocation of eNOS to a detergent-insoluble fraction. The change in eNOS localization, however, did not result in a change in enzyme activity. Inhibition of tyrosine kinases blocked phosphorylation of ENAP-1 and also prevented the translocation of eNOS. We did not observe an alteration in the overall pattern of eNOS intracellular distribution under our experimental conditions. It should be noted, however, that our results represent a “steady-state” condition, because eNO production was monitored over several hours under the various conditions. Therefore, transient changes would not be detected in phosphorylation states or in intracellular location that have been reported by other investigators (Prabhakar et al. 1998).

We found that continuous treatment of endothelial cells with Bk for up to 6 hours stimulated eNO production to about twice the basal level, whereas cotreatment with
vanadate caused a much greater (~20-fold) and synergistic agonist response. The response to vanadate was abolished by the tyrosine kinase inhibitors genistein (Akiyama et al. 1987) and tyrphostin (Gazit et al. 1991), thus demonstrating the specific involvement of tyrosine phosphorylation in this enhancement. We also observed a synergistic enhancement by vanadate of A23187-mediated activation of eNO production, suggesting that the observed vanadate effect was not mediated by Bk.

The tyrosine phosphorylation–associated enhancement of eNO production cannot be explained through an increase in the relative amounts of eNOS because Western blots showed no significant change in the distribution or in total eNOS levels in either particulate or cytosolic fractions after vanadate treatment, consistent with the lack of effect of cycloheximide on enhancement of eNO production by vanadate. The observed vanadate enhancement appears to be indirect, because vanadate had no effect on the specific activity of eNOS when included in an NOS assay of lysates from untreated cells. There was also little difference in the specific activity of eNOS from lysates of cells treated with Bk, vanadate, or Bk/vanadate.

Activation of eNOS may occur within the plasma membrane subcompartments known as caveolae, which are cholesterol-rich structures consisting of a scaffolding protein, caveolin-1, and an array of receptors and signal transduction factors. Caveolin-1 overexpression was found to inhibit eNOS activity in lysates from COS-7 cells coexpressing eNOS, and the effect was reversed by addition of calmodulin (Michel et al. 1997). It has also been reported that eNOS undergoes a flow-mediated dissociation from caveolin and a reassociation with calcium-calmodulin (Rizzo et al. 1998), which could serve to potentiate eNOS activity through maintaining the active dimeric form of the enzyme (Hellermann et al. 1997). Other proteins have been shown to bind to and modify eNOS activity, including ENAP-1 (Venema et al. 1996), the Bk B2 receptor (Ju et al. 1998; Marrero et al. 1999) and hsp90 (Garcia-Cardena et al. 1998; Russell et al. 2000). We found that a 125-kDa tyrosine-phosphorylated protein, and possibly 105- and 75-kDa tyrosine-phosphorylated proteins, were associated with eNOS after treatment with Bk/vanadate. It was previously reported that eNOS coimmunoprecipitated with hsp90 (Garcia-Cardena et al. 1998). Binding of hsp90 is associated with stimulation of eNOS
activity (Garcia-Cardena et al. 1998), and enhanced binding of hsp90 to eNOS also appears to be involved in the estrogen receptor–mediated activation of eNOS (Russell et al. 2000). We found that hsp90 was apparently involved in the vanadate/Bk stimulation of eNO production, on the basis of the inhibition of this stimulation by geldanamycin, a specific inhibitor of hsp90, and on the apparent increase in hsp90 that coimmunoprecipitated with eNOS after exposure to vanadate. It should be noted that hsp90 and ENAP-1 are of the same apparent size and may even be the same protein (R.C. Venema, personal communication, 2000). Thus, the increase in tyrosine phosphorylation of certain proteins appears to trigger a marked enhancement of the activation state of eNOS through association with specific proteins that are presumably involved in regulating eNOS activity in vivo.

Clearly, there are different pathways that may affect eNOS activity, depending on the relative types and levels of phosphorylation/dephosphorylation in a system. Through this regulated interaction of enzymes and accessory proteins, a fluid complex is formed that promotes efficient coupling of the components of the signaling pathway. Our results support the view that protein tyrosine phosphorylation plays a prominent role in the complex signaling network regulating eNOS activity in vivo. This tyrosine phosphorylation–mediated modulation of eNOS activity appears to be due, in large part, to association/dissociation of “modulator” proteins, rather than to a direct modification of tyrosine residues of eNOS.
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Figure 1. Treatment of endothelial cells with sodium orthovanadate causes enhancement of both Bk- and A23187-stimulated NO production. BAECs were incubated with either 2 µmol/L Bk (A) or 1 µmol/L A23187 (B) with or without 50 µmol/L vanadate. Samples were taken at the indicated times and nitrite content was determined. Control, ●; Bk and A23187, ▲; vanadate, ■; Bk/vanadate and A23187/vanadate, Δ; summation of Bk and vanadate or A23187 and vanadate, +.
Figure 2. Dose response for Bk or A23187 on NOS activity with or without sodium orthovanadate. Bk (A) or A23187 (B) at the indicated concentrations was incubated with BAECs for 4 hours in the presence (▲) or absence (●) of 50 µmol/L vanadate. Nitrite content in the culture medium was then determined.
Figure 3. Effects of NOS inhibitors and protein phosphatase or kinase inhibitors on the sodium orthovanadate enhancement of Bk-or A23187-stimulated NO production. BAECs were incubated for 4 hours in the presence of 2 µmol/L Bk (A) or 1 µmol/L A23187 (B). Where indicated, the following compounds were also present: vanadate (V, 50 µmol/L), genistein (Gen, 40 µmol/L), tyrphostin ST638 (Tyr, 20 µmol/L), okadaic acid (OA, 0.5 µg/mL), phenylarsine oxide (PAO, 10 µmol/L), L-NAME (NAME, 1 mmol/L), or dexamethasone (Dex, 50 µmol/L). Samples of culture medium were taken after 4 hours of incubation and nitrite content was determined. Minus sign under left bar indicates control.
Figure 4. Effects of vanadate treatment on caveolar localization of eNOS in endothelial cells stimulated with Bk. BAECs were incubated for 4 hours as indicated with 10 µmol/L Bk, 50 µmol/L sodium orthovanadate (V), or both (Bk/V). Lysates of cells were then subjected to isopycnic density gradient centrifugation and fractionation as described in Methods. Aliquots of the fractions were dot-blotted, and the blot was probed with an eNOS-specific polyclonal antibody. Fraction 1 represents the top and fraction 10, the bottom of the gradient. The caveolar peak corresponds to fraction 3, as determined by immunoblot analysis with anti–caveolin-1 (not shown).
Figure 5. Effect of vanadate on phosphotyrosine-containing proteins in Bk-stimulated endothelial cells. BAECs were incubated for 4 hours with 2 µmol/L Bk in the presence or absence of 50 µmol/L vanadate (V). Cell lysates were prepared, immunoprecipitated with anti-eNOS monoclonal antibody, and run on SDS PAGE. Blots were probed with an anti-phosphotyrosine monoclonal antibody (left) and anti-eNOS monoclonal antibody (right). C indicates control.
Figure 6. Immunoprecipitation of Bk-treated endothelial cell lysates with anti-eNOS monoclonal antibody. BAECs were incubated for 4 hours with 2 µmol/L Bk in the presence or absence of 50 µmol/L vanadate (V), followed by preparation of cell lysates and immunoprecipitation with an anti-eNOS monoclonal antibody. Immunoprecipitated proteins were separated by SDS PAGE and immunoblotted with the indicated monoclonal antibodies. C indicates control.
References


Appendix A (Continued)

Endothelial Argininosuccinate Synthase mRNA 5´-Untranslated Region Diversity: Infrastructure for Tissue-Specific Expression*

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Abstract

Based on the integral role that argininosuccinate synthase (AS) plays in the production of nitric oxide in vascular endothelial cells and urea in liver, an analysis was carried out to determine whether signals reside in the AS mRNA to account for tissue differences in AS function and location. Reverse transcriptase-PCR and sequence analysis showed that the AS mRNA coding region was the same for both endothelial cells and liver; however, 5′-RACE analysis (rapid amplification of cDNA ends) identified AS mRNA species in endothelial cells in addition to a major 43-nucleotide (nt) 5′-untranslated region (UTR) AS mRNA with overlapping extended 5′-UTRs of 66 and 92 nt. Comparison to the genomic sequence immediately upstream of the reported transcription start site for the human and mouse AS gene suggested that expression of all three species of bovine endothelial AS mRNA are driven by a common promoter and that 5′-UTR diversity in endothelial cells results from three transcriptional initiation sites within exon 1. RNase protection analysis and real-time reverse transcriptase-PCR verified and quantitated the differential expression of the extended 5′-UTR species relative to the major 43-nt 5′-UTR AS mRNA. In vitro translation studies showed a less pronounced but similar discordant expression. Sequential deletions starting from the 5′-UTR terminus of the 92-nt 5′-UTR construct resulted in a corresponding increase in translational efficiency, but the most pronounced effect resulted from mutation of an upstream open reading frame, which restored translational efficiency of the 92-nt 5′-UTR AS mRNA. When the different AS mRNA 5′-UTRs, cloned in front of a luciferase reporter gene, were transfected into endothelial cells, the pattern of luciferase expression was nearly identical to that observed for the different 5′-UTR AS mRNAs in endothelial cells. Given the different roles ascribed for argininosuccinate synthase, urea versus NO production, these results suggest that sequence in the AS gene represented by position -92 to 43 nt from the translation start site in the extended AS mRNA 5′-UTRs plays an important role in differential and tissue-specific expression.
Appendix B (Continued)

The abbreviations used are: AS, argininosuccinate synthase; UTR, untranslated region; nt, nucleotide; ORF, open reading frame; uORF, upstream ORF; AL, argininosuccinate lyase; BAEC, bovine aortic endothelial cells; RACE, rapid amplification of cDNA ends; RPA, ribonuclease protection assay; RT, reverse transcriptase; TBST, Tris-buffered saline Tween 20; NO, nitric oxide.
Argininosuccinate synthase (AS) catalyzes the reversible ATP-dependent ligation of citrulline and aspartate to produce argininosuccinate, AMP, and inorganic pyrophosphate. The primary role of AS is the detoxification of ammonia via the urea cycle in the liver (Morris 1992). Although this essential physiological function occurs in the liver and to a lesser extent in the small intestine, virtually all other mammalian tissues possess detectable levels of AS and a second urea cycle enzyme argininosuccinate lyase (AL). Together these two enzymes have the net effect of generating arginine from citrulline and aspartate. In the kidney, AS and AL are responsible for the de novo synthesis of arginine to be released into the bloodstream (Wu et al. 1998). In other tissues, however, the function of this metabolic pathway remained obscure until the discovery of arginine-derived nitric oxide (NO) (Hecker et al. 1990).

In endothelial cells, AS catalyzes the rate-limiting step (Xie et al. 1997) in the synthesis of L-arginine from L-citrulline. Endothelial nitric-oxide synthase then utilizes the arginine, converting it back to citrulline to produce NO. Thus, available arginine is a prerequisite for NO production. Interestingly, intracellular levels of arginine in endothelial cells have been estimated to range from 0.1 to 0.8 mM (Baydoun et al. 1990; Block et al. 1995; Gold et al. 1989; Harrison 1997; Hecker et al. 1990; Mitchell et al. 1990; Xie & Gross 1997), well above the reported \( K_m \) of 5 \( \mu \)M for endothelial NO synthase (Harrison 1997). Yet an increase in extracellular L-arginine levels and/or an increase in the synthesis of arginine from extracellular citrulline will increase NO production from stimulated endothelial cells (Flam et al. 2001; Greene et al. 1993; Hecker et al. 1990; McDonald et al. 1997a; McDonald et al. 1997b; Shuttleworth et al. 1995; Xie et al. 2000). Moreover, during shear stress-induced NO synthesis (Dimmeler et al. 1999) AS levels are up-regulated along with several other genes suggested to play a role in the regulation of NO production (McCormick et al. 2001). These results have been taken to suggest that a separate pool of arginine is maintained for NO production in endothelial cells by either transport and/or the regeneration of arginine from citrulline (Flam et al. 2001; Greene et al. 1993; Hecker et al. 1990; McDonald et al. 1997a; McDonald et al. 1997b; Shuttleworth et al. 1995; Xie et al. 2000). The physiological importance of the arginine regeneration system also was suggested by a case report of
two infants with a deficiency in argininosuccinate lyase (also essential for arginine regeneration from citrulline) who were shown to be hypertensive. Infusion of arginine resulted in the lowering of blood pressure in these infants, suggesting a critical role for arginine regeneration in the regulation of systemic blood pressure (Fakler et al. 1995).

More recently we have shown that AS and AL co-fractionate (along with endothelial NO synthase) with the endothelial caveolar fraction (Flam et al. 2001); therefore, regeneration of arginine from citrulline is not only integral in endothelial NO production but also may involve different regulation and cellular localization relative to the hepatic AS and urea production. For this reason we have proposed that an endothelial isoform expressed from the same gene as the liver form (Freytag et al. 1984b) manifests some variations allowing AS to function in a different cellular location (Flam et al. 2001) and to be coordinately regulated with NO production (McCormick et al. 2001). In support of this hypothesis, variations in the physical properties of the liver and endothelial cell AS protein were detected using different physical separation techniques and visualization by immunoblotting.

Based on these findings, we examined AS cDNA from liver and vascular endothelial tissue to determine whether differences in expression and in functional and physical properties could be accounted for at the level of the messenger RNA. In this report we show that the translatable sequence of AS mRNA is identical for both tissues but that the sequence represented by the diversity of endothelial AS mRNA 5´-UTRs may account in part for the specialized regulation of expression in nitric oxide production.
Experimental Procedures

Cell Culture and RNA Isolation — Bovine aortic endothelial cells (BAEC) were cultured in Dulbecco’s modified Eagle’s medium (Mediatech) supplemented with 10% fetal bovine serum (HyClone Laboratories), penicillin, streptomycin, and amphotericin B (Mediatech). Total RNA was isolated from BAEC by the method of Chomczynski and Sacchi (Chomczynski et al. 1987) using Tri Reagent (Molecular Research Center) according to the manufacturer’s protocol. Total RNA from bovine liver was purchased from CLONTECH Laboratories.

Reverse Transcriptase-Polymerase Chain Reaction — Total RNA from bovine liver and BAEC was reverse-transcribed using Superscript II recombinant Moloney murine leukemia virus reverse transcriptase (Invitrogen). The annealing reaction, 10 µg of total RNA and 100 ng of random hexamers, was incubated at 65 °C for 10 min and then cooled to 4 °C. 1x first strand buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2), 10 mM dithiothreitol, 40 units of RNasin, 500 µM dNTP each, and 500 units of the Superscript RT were added, and the reaction was incubated at 37 °C for 1 h. The reaction was stopped by incubation at 80 °C for 10 min. The resulting cDNA was amplified by PCR using primers (Integrated DNA Technologies) designed against the published bovine liver AS sequence (M26198). Primer sequences are listed in Table I. ASL10 was combined with ASR1338, ASR773, and ASR348 to yield fragments of 1328, 763, and 338 bp, respectively. ASL572 was combined with ASR1209 to yield a 637-bp fragment, and ASL882 was combined with ASR1338 to yield a 456-bp fragment. Primers were named based on the designation of position 1 as the first base of the AUG start codon.

All PCR reactions contained 1x assay Buffer B (10 mM Tris-HCl, pH 8.3, and 50 mM KCl), 200 µM each dNTP, 1.5 mM MgCl2, 10 µl of cDNA, 5% Me2SO, 50 pmol of each primer, and 2.5 units of Taq polymerase (Fisher Scientific) and consisted of 35 cycles at 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 3 min. Reactions were started by a 5-min denaturation step and ended with a 10-min synthesis step. PCR fragments were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.
Rapid Amplification of cDNA Ends (RACE) RT-PCR—5′-and 3′-RACE analysis was carried out using the SMART RACE cDNA amplification kit (CLONTECH Laboratories). This technique involved the incorporation of a “Smart Oligo” onto the 5′-end of the reverse-transcribed cDNA for the 5′-RACE analysis. For the 3′-RACE analysis, the Smart Oligo was attached to an oligo (dT) primer to yield cDNA that had a complete 3′-UTR, a poly-A tail, and a Smart Oligo sequence extension. The 5′-and 3′-ends of AS were amplified using a standard PCR protocol. Primers were designed so that a short section of the coding region was amplified along with the 5′-or 3′-UTR. ASR348 was combined with the Smart Oligo primer to amplify the 5′-end, and ASL1188 was combined with the Smart Oligo primer to amplify the 3′-end. Primer sequences are listed in Table I. Amplified 5′-and 3′-RACE fragments were cloned and sequenced. To increase the efficiency of recovery of the specific RACE clones colony lifts were performed, and positive clones were selected by hybridization to the 1328-bp AS cDNA fragment generated by RT-PCR.

Ribonuclease Protection Assays (RPAs)—RPAs were performed using the RiboQuant multiprobe RNase protection assay system (BD Pharmingen). A portion of the AS 5′-UTR and 5′-coding region was amplified by RT-PCR using primers ASL-82 and ASR94 (listed in Table I) and used as a template to produce the RPA probe. The 176-bp fragment was cloned using the TOPO TA cloning dual promoter kit (Invitrogen) and sequenced. To generate the antisense RNA probe, the plasmid construct containing the 176-bp fragment was linearized with XhoI, purified by phenol/chloroform extraction and ethanol precipitation, and quantitated. The 32P-labeled RNA probe was synthesized using T7 RNA polymerase and hybridized to 100 µg of BAEC total RNA or 15 µg of liver total RNA following the manufacturer’s protocol. RNase digests were performed as per the standard protocol except that the RNase A was optimized to a final concentration of 38 ng/µl. Protected fragments were analyzed on a 6% acrylamide, 7 M urea sequencing gel. The gel was dried and exposed to film. Band densities were quantitated using ImageQuant software (Molecular Dynamics).

Real-time RT-PCR—Total RNA from bovine liver and BAEC was DNase-treated using DNA-free DNase treatment and removal reagent (Ambion) following the
manufacturer’s protocol. 1 µg of RNA was reverse-transcribed with the Superscript first-strand synthesis system for RT-PCR (Invitrogen) following the protocol for high G-C content mRNAs. An AS-specific primer, ASR348, was used to reverse transcribe, and the resulting cDNA was amplified using the SYBR Green PCR master mix (Applied Biosystems). PCR conditions were 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The PCR products were detected in real time using the iCycler iQ detection system (Bio-Rad). Primer sets were designed to amplify regions of AS mRNA common to all species, ASL228 and ASR278, as well as to detect the extended 5´-UTRs of 66-nt ASL-62 and ASR-12 and 92-nt ASL-84 and ASR-34 (Table I). AS mRNA was quantitated relative to a standard curve of AS plasmid DNA generated for each primer set. Replicates of five were performed for each primer set with each RT reaction. Results were reported as a percentage of the total AS mRNA.

**Coupled in Vitro Transcription/Translation** — Full-length AS cDNA was constructed to contain the 92-nucleotide 5´-UTR and subcloned into the vector pPDM-2 (Epicentre Technologies). AS cDNA was amplified by PCR using Pfu Turbo DNA polymerase (Stratagene) to contain each of the three 5´-UTRs. 5´-primers contained a BamHI site, T7 promoter sequence, spacer region, and 20 bases of AS cDNA sequence corresponding to the first twenty bases of each 5´-UTR species (Table II). A control 5´-primer contained a BamHI site, T7 promoter sequence, spacer region, and a Kozak sequence of six bases (21) followed by the ATG and 20 bases of AS cDNA sequence downstream of the start codon. The 3´-primer used for amplification of the full-length AS cDNA was ASR1338 (Table I). The PCR products were gel-purified, quantitated, and then transcribed and translated using the TNT T7 Quick for PCR DNA transcription/translation kit (Promega). For detection, Transcend biotin-lysyl-tRNA (Promega) was added to the reaction, resulting in the incorporation of biotinylated lysine into the translated protein.

**In Vitro Translation of Capped AS mRNA** — PCR fragments containing four different 5´-UTRs (three different AS UTRs plus the control, all with the T7 promoter sequence incorporated) were subcloned into the pPDM-2 vector using the BamHI site engineered into the fragments at the 5´-end and an EcoRI site at the 3´-end. Constructs
were digested with EcoRV at a site past the 3´-end to prevent run-on transcription. Template DNAs were transcribed using T7 RNA polymerase with the addition of Ribo m7G cap analog (Promega) following the manufacturer’s protocol recommended for m7G cap incorporation. Transcribed RNA was DNase-treated and purified using minispin G50 Sephadex (CPG) columns. The Flexi Rabbit reticulocyte lysate system (Promega) was used for the translation reaction following the manufacturer’s protocol with the addition of 0.75 µl of transcend tRNA and 1 µg of capped RNA. KCl conditions were optimized to 40 µM.

**Western Blotting Analysis** — Translated proteins were separated by SDS-PAGE on 12% Tris-HCl Ready Gels (Bio-Rad). Following electrophoresis, proteins were transferred to Immobilon-P membranes (Millipore) using a wet transfer method (Bio-Rad). Following a blocking step of 1 h in TBST (20 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Tween 20), the membranes were incubated for 1 h at room temperature with Streptavidin-horseradish peroxidase antibody (Promega or Jackson Immuno-Research Laboratories) at a final concentration of 0.025 µg/ml. The membranes were then washed three times in TBST and three times in nanopure water for 5 min per wash. The in vitro-translated proteins were visualized using an enhanced chemiluminescent reagent according to the manufacturer’s protocol (Amersham Biosciences) or Transcend reagent (Promega). Band densities were quantitated using ImageQuant software (Molecular Dynamics). Membranes were then placed in blocking buffer composed of 5% nonfat dry milk in TBST for 1 h at room temperature. Membranes were incubated with the primary antibody, anti-ß-actin, mouse monoclonal, clone AC-15 (Sigma) in blocking buffer for 1 h at room temperature. Membranes were washed and incubated with the secondary antibody, peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) in blocking buffer for 1 h at room temperature. After washing with TBST, membranes were visualized using chemiluminescent reagent and exposed to film. Band densities were quantitated as before.

**Mutational Analysis** — Deletion mutants were constructed to include 86, 81, 76, and 71 nt of the AS 5´-UTR along with the T7 promoter sequence by amplification and subcloning as described previously. Primer sequences are listed in Table II. Mutations of
the uORF AUG were carried out using a PCR strategy. A sense primer, ASL-70 Mut1, was designed to generate a single base mutation, converting the uAUG to AAG. A second primer, ASL-73 Mut2, was designed to alter the context of the uAUG by changing the surrounding nucleotides. The sequence GGG AUG CGC was mutated to AGG AUG GGC, changing critical nucleotides at position -3 and +4 relative to the AUG to improve the function of the uAUG (Kozak 1987). These primers were combined with ASR429 to amplify fragments of 499 and 502 bp, following the protocol listed in the PCR section. PfuTurbo DNA polymerase (Stratagene) was used to reduce the frequency of error in the amplification. The amplified fragments were gel-purified, sequenced, and used as right primers in a second amplification with ASL-92T7. Fragments were digested with BamHI and NarI and subcloned into the existing 92-nt 5´-UTR full-length AS cDNA construct. The deletion constructs and mutant AUG constructs were transcribed, translated, and analyzed by Western blotting.

Transfection Analysis — Luciferase reporter constructs were designed to include each of the AS 5´-UTRs cloned directly after the simian virus 40 promoter and before the start codon of the luciferase gene. Left primers ASL-92HindIII, ASL-66HindIII, and ASL-43HindIII were combined with ASRLuc (Table I) to amplify the different sized AS 5´-UTRs with a HindIII site on the 5´-end and 45 bases of the luciferase gene attached to the 3´-end. This strategy took advantage of a NarI site within the luciferase gene close to the start codon. The amplified fragments were digested with HindIII and NarI and cloned into pGL3Control in place of the existing 5´-UTR. Constructs were verified by sequencing. BAEC to be used for transfections were plated at 2 x 10⁴ cells per well in a 24-well plate. Control plasmids (Promega) included pGL3Control as a positive control, pGL3Basic as a promoterless negative control, and pRL-TK, a renilla expression vector, as an internal transfection control. Control, Basic, and experimental plasmids (200 ng each) and pRL-TK (50 ng) were transiently transfected into BAEC using Transit-LT1 (Panvera) in serum-free medium. After 4.5 h, the medium was replaced with complete medium, and cells were cultured for 48 h. Lysates generated with Passive lysis buffer (Promega) were assayed for luciferase and renilla activity using Promega’s Dual-Luciferase Reporter Assay System according to the manufacturer’s recommendations.
Luciferase and renilla activity were measured as relative light units using a luminometer (Turner Designs). Experiments were carried out three times in triplicate. Luciferase expression was normalized to renilla activity.
Results

*Comparison of RT-PCR Fragments from Bovine Liver and Endothelial Cell AS mRNA* — To investigate possible differences in the coding region of AS mRNA, total RNA from bovine liver and cultured bovine aortic endothelial cells was reverse-transcribed and amplified by PCR using a series of oligonucleotide primer sets designed to cover the entire coding region of the mRNAs. The resulting amplified fragments were separated by agarose gel electrophoresis, shown in Fig. 1. As can be seen, the relative mobility of each set of amplified fragments was identical, indicating that there were no substantial differences in the two AS messages that would indicate splicing variations of the ~13 exons that define the AS gene (Freytag et al. 1984a). To confirm this finding sequence analysis was carried out, which showed that there were no differences (data not shown) within the translatable sequence of endothelial and liver AS mRNA.

*RACE Analysis of the 5´-and 3´-Ends of Liver and Endothelial Cell AS mRNA* — Because the UTRs of mRNAs can influence stability, localization, and translation, rapid amplifications of the reverse-transcribed 5´- and 3´-ends of AS mRNA were carried out on bovine liver and cultured endothelial cell total RNA. The products of the RACE analyses were subcloned and sequenced.

For the 3´-RACE analysis, minimal differences attributed to minor polymorphisms at the level of the gene were observed. Direct sequence analysis further confirmed this finding (data not shown). In contrast, the 5´-RACE analysis of endothelial AS mRNA yielded clones containing different length extensions of the 5´-UTR. The majority of clones represented AS mRNA with 43 nucleotides of sequence upstream of the AUG start codon common to liver and endothelial cells (Fig. 2). A single isolated clone contained the identical 43 nt in the 5´-UTR but with an additional 23 nt of upstream sequence. A second clone verified the sequence of the 66 nt 5´-UTR but contained an additional 26 nt of upstream sequence for a total of 92 nt of 5´-UTR. Importantly, the 66- and 92-nt 5´-UTR clones were only identified by 5´-RACE analysis with endothelial cell total RNA. 5´-RACE analysis of liver total RNA yielded only the 43 nt AS 5´-UTR.

*Extended 5´-UTR Sequence Comparison* — To determine the possible genomic origin of the endothelial-specific AS 5´-UTR species, these sequences were compared
with the 5’-UTR and genomic 5´-flanking regions from human (Jinno et al. 1985) and mouse (Surh et al. 1991) (Fig. 3). Comparison of the 43-nt bovine AS mRNA 5´-UTR with exon 1 from human and mouse AS gene sequences demonstrated 72 and 64% homology, respectively. When a comparison was made of the 92-nt 5´-UTR from endothelial AS mRNA with the 5´-flanking genomic sequence from human and mouse, the percentage of homology increased to 78 and 71%, respectively. A sequence of 10 nt surrounding the TATTA box was identical in all three species. Importantly, the upstream AUG found in the extended 5´-UTR AS mRNAs from bovine endothelial cells was distinctly identified in the 5´-flanking genomic regions of both human and mouse AS genes.

Ribonuclease Protection Assays of the 5´-UTR of Liver and Endothelial Cell AS mRNA — To verify and quantitate these results, RPAs were carried out on total RNA isolated from BAEC and liver using an antisense RNA probe complementary to the AS sequence extending from 82 nt upstream of the AUG start codon to 94 nt downstream of the start codon. Consistent with the 5´-RACE analysis, RNase protection analysis of the endothelial RNA yielded three different sized fragments (Fig. 4) corresponding to the predicted sizes of the different 5´-RACE AS mRNA sequences (43, 66, and 92 nt) identified previously. Quantitation by densitometry showed that 78% of the total AS mRNA contained the shortest 43-nt 5´-UTR sequence followed by 12% for the 92-nt 5´-UTR sequence and 10% for the 66-nt 5´-UTR. Notably, the RPA for liver AS mRNA only demonstrated the existence of the 43-nt 5´-UTR sequence.

Real-time RT-PCR Analysis of the 5´-UTR of Liver and Endothelial Cell AS mRNA — A second approach to quantitate relative levels of 5´-UTR AS mRNA species took advantage of the increased sensitivity of real-time RT-PCR analysis. As shown in Fig. 5, real-time RT-PCR analysis corroborated the RPA results, demonstrating again the disproportionate expression of three AS mRNA 5´-UTR species found in endothelial cells. Because of the linear response of the technique over a large dynamic range, more accurate quantitation was generated for the percentages of each mRNA species. Plasmid DNA standards were detected in duplicate over a range of 9 orders of magnitude, easily allowing for the precise quantitation of mRNA levels differing by a factor of 100 or
more. The percentage of the 43-, 66-, and 92-nt 5′-UTR AS mRNA species were determined to be 93.79 ± 1.24, 3.75 ± 1.70, and 2.46 ± 0.93%, respectively, of the total AS mRNA. The percentages are lower than those detected by ribonuclease protection analysis, probably because of the limitations of quantitation from x-ray film over a wide range of signal. Although the 66-and 92-nt 5′-UTR species were detected in liver above negative controls, levels were significantly lower than in endothelial cells, representing less than 0.1 ± 0.06% of total liver AS mRNA. Real-time RT-PCR analysis also confirmed differences in AS mRNA expression relative to total RNA, showing that in liver there is an ~40-fold higher level of expression compared with endothelial cells.

*In Vitro Translational Efficiency of the 5′-UTR AS mRNAs* — Sequence analysis of the diverse 5′-UTR AS mRNAs showed that each contained >70% G+C content and that the two longer forms contained a uORF frame. These features have been noted by other investigators to affect the translational efficiency of mRNAs (Kozak 1991). For this reason, experiments were designed to examine the influence that the diversity of endothelial AS 5′-UTRs might have on the translation of AS mRNA.

Full-length AS mRNA containing the 92-nt 5′-UTR sequence was cloned and then amplified to yield each of the other AS mRNA 5′-UTR species. An additional clone of AS mRNA referred to as the control was constructed to contain a 5′-UTR with optimal features relative to AUG start codon recognition and initiation (Kozak 1987). These constructs were transcribed and translated initially in a coupled transcription/translation system that visualized the protein products by immunodetection of biotinylated lysine incorporated during translation. Protein expression, analyzed by SDS-PAGE and Western blotting, showed that the AS mRNA with the shortest 43-nt upstream sequence translated as well or better than the control. The most dramatic effect on translational efficiency was observed with the longest endothelial 5′-UTR of 92 nt. A protein product was essentially undetectable even after longer exposures, whereas the intermediate length 5′-UTR of 66 nt translated nearly as well as the 43-nt 5′-UTR (data not shown).

Because the coupled system did not produce capped (7-methylguanosine, m7G) AS mRNA, the experiments were repeated using AS mRNA that contained a 5′-terminal m7G cap. The translation products containing biotinylated lysine generated from the
different AS 5′-UTR mRNA species were separated by SDS-PAGE and visualized by immunodetection (Fig. 6). This time using capped AS mRNA, a product for the 92-nt 5′-UTR AS mRNA was detectable, probably reflecting the increased efficiency of translation observed for each of the capped species examined. The intermediate length 5′-UTR of 66 nt translated nearly as well as the control containing the Kozak sequence (Kozak 1991); however, the 43-nt 5′-UTR construct translated more efficiently than either the 66-nt 5′-UTR construct or the control. Examining the comparative level of AS protein produced, where the ratio to control remained essentially identical to that observed using uncapped AS mRNA, supported this conclusion. These differences demonstrated that the 5′-UTR species of endothelial AS mRNA influenced in vitro translational efficiency.

**Deletion and Mutational Effects on Endothelial 5′-UTR AS mRNAs Translational Efficiency** — To determine the basis for the decreased translational efficiency of the longer 5′-UTR, deletion and mutational analysis was carried out. As shown in Fig. 7, sequential deletion of the 92-nt 5′-UTR yielded results demonstrating a direct correlation of 5′-UTR length with translational efficiency. However, mutation of the upstream AUG to eliminate the uORF restored the translational efficiency of the 92-nt 5′-UTR AS mRNA to essentially that observed for the 66-nt 5′-UTR species. When the context of the upstream AUG was mutated to conform to an optimal consensus Kozak sequence, there was no significant decrease in translational efficiency observed with the 92-nt 5′-UTR containing AS mRNA. These results suggest that the uORF is an important feature affecting translational efficiency and that at least 22 nt of sequence 5′ to the uORF are required for maximum suppression.

**Effects of AS mRNA 5′-UTRs on Luciferase Expression in Transfection Studies** — To isolate the effects of the different AS mRNA 5′-UTRs on expression, each 5′-UTR sequence was cloned in front of a luciferase reporter gene. Constructs were transfected into BAEC, and luciferase expression measured. Relative to the 43-nt 5′-UTR construct, expression of the 66 and 92-nt 5′-UTRs were 8-and 6-fold lower, respectively (Fig. 8). These results were essentially consistent with the discordant expression of the various 5′-UTR AS mRNAs in endothelial cells, demonstrating that the sequence from position -92
Appendix B (Continued)

to -43 in the AS gene relative to the translational start codon is sufficient to affect
differential expression of the three 5'‐UTR AS mRNA species in endothelial cells.
Discussion

Although the physiological role for AS was originally identified with the urea cycle in the liver, virtually all other mammalian tissues possess detectable levels of AS and a second urea cycle enzyme, AL. In nitric oxide-producing tissue, these two enzymes together have the net effect of regenerating arginine from citrulline and aspartate, providing arginine for NO production (Wu & Morris 1998). We had previously shown that endothelial AS differs from liver AS, not only in its relationship to NO production but also by its caveolar co-localization with nitric oxide synthase (Flam et al. 2001).

Based on these findings, we examined whether an endothelial isoform of AS may exist that is encoded by the same respective hepatic urea cycle AS gene (Freytag et al. 1984b) but that plays a specialized role in regenerating arginine from citrulline for NO production. Although physical differences (mobility and isoelectric point) were observed (B. R. Flam, L. C. Pendleton, B. L. Goodwin, L. P. Solomonson, and D. C. Eichler, unpublished observations), a comparison of the coding regions of liver and endothelial AS mRNA by RT-PCR analysis suggested that alternative splicing could not account for these physical differences or for the functional and localization differences. This result was further confirmed by direct sequence analysis.

Expression of genes that play a key role in metabolic processes are often regulated at multiple levels, and differences in regulation and localization have been attributed in some cases to differences in UTRs of an mRNA (de Moor et al. 2001; Gray et al. 1994; Gray et al. 1998; Kozak 2000; Sonenberg 1993; van der Velden et al. 1999). We therefore compared sequences of both the 5′-and 3′-UTR of AS mRNA from liver and endothelial cells to assess the possible importance of these regions in the regulation of AS expression in endothelial cells. In this report we have shown that AS mRNA from bovine liver and bovine aortic endothelial cells differs in the relative level of expression and in the diversity observed for the 5′-UTR region. 5′-RACE analysis demonstrated unique expression of three different 5′-UTR AS mRNAs in endothelial cells. In contrast, only the shortest 5′-UTR of 43 nt was detected in liver.

Sequence inspection of the various 5′-UTRs of AS mRNA defined by 5′-RACE, RT-PCR, RNase protection analysis, and real-time RT-PCR highlighted the following
features. First, the expression of the various AS mRNA 5'-UTR species is
disproportionate. The AS mRNA with the shortest 43-nt 5'-UTR represents the major
form (~94%) of the total AS mRNA, whereas the longer 5'-UTRs of 66 and 92 nt
represent ~4 and 2%, respectively. Second, all 5'-UTRs for endothelial AS mRNA fall
within the average length of eukaryotic 5'-UTRs (50–100 nt) (Kozak 1987). Third, an
upstream AUG codon was detected in the 66- and 92-nt extended 5'-UTRs that is out-of-
frame with the downstream AUG start codon but in-frame with multiple ORFs in the
coding sequence of AS mRNA. Finally, composition analysis revealed that all three 5'-
UTR sequences are highly enriched in G+C content (~76%), suggesting that all are
capable of forming stable complex secondary structures known to affect translational
efficiency (Kozak 2000).

Sequence analysis also suggested the possible origin of the 5'-UTR AS mRNA
species. Comparison to the 5'-UTRs from both human (Jinno et al. 1985) and mouse
(Surh et al. 1991) AS mRNA demonstrated a high degree of homology. This similarity
increased when the extended 5'-UTR sequences from bovine endothelial AS mRNA were
compared with the genomic sequence immediately upstream of the reported transcription
start site for the human and mouse AS gene. Our findings suggest that expression of all
three species of bovine endothelial AS mRNA are driven by a common promoter, and
that 5'-UTR diversity in endothelial cells results from three transcriptional initiation sites
within exon 1.

Considerable research related to the 5'-UTR region of mRNAs has suggested a
regulatory role in translational efficiency in part through cis effects at the primary (e.g.
upstream short ORFs affecting ribosome scanning), secondary (e.g. hairpins and stem-
loop structures), or tertiary (e.g. pseudoknots) levels (Gray & Hentze 1994; Gray &
1993; van der Velden & Thomas 1999). Such effects may also involve the contribution of
RNA-binding proteins (Morris & Geballe 2000). Some general principles by which
uORFs have been proposed to participate in translational control include the process of
recognition of uORFs, regulation of reinitiation at downstream cistrons after translation
of uORFs, and regulatory effects of peptides encoded by uORFs (Morris & Geballe 2000).

In endothelial cells there are only two species of AS mRNA that contain an identical uORF, and they represent at most no more than 6% of the total AS mRNA. But because these features are regarded as important determinants of translational efficiency, this suggested that the diversity at the 5′-end of AS mRNA transcripts may have important biological implications on the regulation of translation and may possibly explain observed differences between steady-state mRNA levels and protein expression.

For this reason, after defining the diversity of 5′-UTR AS mRNAs in endothelial cells, an examination was carried out to determine to what extent the various 5′-UTRs of AS mRNA influence the efficiency of translation. Results from these experiments clearly showed that the nature of the AS mRNA 5′-UTR influenced translation. Based on the in vitro translation results for the 92-nt 5′-UTR, we expected that the intermediate length 66-nt 5′-UTR that still contained the uORF would yield similar results. However, the 66-nt 5′-UTR AS mRNA expression was nearly 70% of that observed for the 43-nt 5′-UTR AS mRNA. Improvement in the translational efficiency of all AS mRNA 5′-UTRs was observed in vitro when the message was capped, although disproportionate expression was still evident for the 92-and 66-nt 5′-UTR AS mRNAs. We had not anticipated that the control in these experiments, which possessed no AS mRNA 5´-UTR and was constructed to contain what may be considered an optimal consensus sequence around the start codon, would not express protein as well as the 43-nt 5′-UTR AS mRNA. This result indicated that features of the 43-nt 5′-UTR as well as features in the translated sequence of AS mRNA compensate for the lack of optimal consensus sequence within the context of the AUG start codon (Kozak 1991). The fact that loss of the uORF restored the translational efficiency of the 92-nt 5′-UTR to that observed for the 66-nt 5′-UTR and that the 66-nt 5′-UTR containing this uORF expressed protein at ~70% of the 43-nt 5′-UTR also suggested that attenuation of translation required ~22 nt of sequence 5′- to the uORF for maximum effect.

Transfection studies, which focused on the effect of AS 5′-UTR sequence diversity on luciferase expression in endothelial cells, produced results essentially
consistent with the levels of expression observed in endothelial cells for the different 5´-UTR AS mRNAs. These findings suggested that sequence in the AS gene encoding positions -92 to -43 in the extended 5´-UTRs of AS mRNAs was sufficient to affect differential expression.

In summary, there is a remarkable diversity and disproportionate expression of each of the 5´-UTR AS mRNA transcripts that clearly differentiate AS expression in vascular endothelial cells from that in liver. We have demonstrated that expression of a 43-nt 5´-UTR AS mRNA is robustly enhanced in liver versus endothelial cells and that the unique diversity of 5´-UTR AS mRNAs in endothelial cells reflects sequence in the AS gene that permits selective modulation and tissue-specific expression.
Acknowledgments

We thank Madolyn L. Bowman and Luis L. Alvarado, University of South Florida Honors undergraduate students who participated in this research. We also thank the Molecular Biology Core Facility at the H. Lee Moffitt Cancer Center and Research Institute for support.
Table I. Primer Sequences for PCR Amplification of AS

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer Sequence</th>
<th>Name</th>
<th>Primer Sequence</th>
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<tr>
<td>ASL10</td>
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<td>ASR1209</td>
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<td>ASRluc</td>
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<td>ASL-62</td>
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Sequence written in 5' -> 3' notation. L and R designate sense and antisense primers. Primers with "*" denote sequence in the 5'-UTR and increasing numbers indicate sequence upstream from the start AUG. The nucleotides changed by the mutagenic primers are in bold type.
Table II. Primer Design for *In Vitro* Transcription and Translation

<table>
<thead>
<tr>
<th>Primer Name</th>
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<th>T7 Promotor Sequence</th>
<th>Spacer Region</th>
<th>AS specific sequence</th>
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<td>TAA TAC GAC TCA CTA TAG GG</td>
<td>A ACA G</td>
<td>CC CTG CCC CCC GGC CCC GAG</td>
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<tr>
<td>ASL-66T7</td>
<td>GGA TCC</td>
<td>TAA TAC GAC TCA CTA TAG GG</td>
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<td>AC CCG GGA TGC GCG CCG AAA</td>
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<tr>
<td>ASL-43T7</td>
<td>GGA TCC</td>
<td>TAA TAC GAC TCA CTA TAG GG</td>
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<td>GC CCT GCT CCG CCG ACT GCT</td>
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<tr>
<td>ASL+1T7 Control</td>
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<td>TAA TAC GAC TCA CTA TAG GG</td>
<td>A GCC ACC</td>
<td>ATG TCC GGC AAA GGC TCC GTG GT</td>
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<tr>
<td>*ASL-86T7</td>
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<td>*ASL-71T7</td>
<td>GGA TCC</td>
<td>TAA TAC GAC TCA CTA TAG GG</td>
<td>A ACA G</td>
<td>TT ATA ACC CGG GAT GCG CCG</td>
</tr>
</tbody>
</table>

Sequence written in 5'->3' notation. Primers with "*" denote sequence in the 5' -UTR and increasing numbers indicate sequence upstream from the start AUG. The Kozak consensus sequence (23) for the control primer is shown in bold type. *Indicates primers designed for deletion mutants.
Figure 1. Amplification of reverse-transcribed AS mRNA from bovine liver and cultured endothelial cells by polymerase chain reaction. A, RT-PCR products from endothelial cells (E) and liver (L) fractionated on a 1.5% agarose gel. Numbers on the left designate size (bp) of molecular standards. B, expected fragment sizes (bp) and location with respect to AS mRNA (bottom).
Appendix B (Continued)

Figure 2. Novel 5’-UTRs of endothelial AS mRNA. The sequence for each AS mRNA 5’-RACE product as well as the normal start codon (boldface) are indicated by a label and an arrow above the sequence. The upstream out-of-frame start codon (boldface) and the uORF are underlined.
**Figure 3. Extended 5′-UTR sequence comparisons.** Sequences from the bovine AS mRNA 5′-UTRs compared with genomic AS 5′-flanking regions and 5′-UTRs from human and mouse. Bovine transcription start sites as well as the translational start codon are indicated by an arrow and a label above the sequence. The AUG and uAUG are shown in boldface type. The underlined sequence denotes human and mouse exon 1. Boxed areas indicate sequence with 100% homology between the three species.

<table>
<thead>
<tr>
<th>Bovine</th>
<th>Human</th>
<th>Mouse</th>
</tr>
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<td>~~~~CCCTGC CCCCUGGCAC CGAGCTTATA ACCCGGGAATG</td>
<td>CCGGCCCTGC CCCCUGGCCC TGGCTTATA ACCCTGGGATG</td>
<td>TTCCTGCCC CCCCAGGCC CTTGCTTATA ACCCTGGGATG</td>
</tr>
<tr>
<td>§92 nt 5′-UTR</td>
<td>§66 nt 5′-UTR</td>
<td>§43 nt 5′-UTR</td>
</tr>
<tr>
<td>Bovine</td>
<td>Human</td>
<td>Mouse</td>
</tr>
<tr>
<td>CGCGCCGAAGA CCCCCCTGAC TCCGCCGACT GCTGCGGCC</td>
<td>GGCACCCTCG CCAGTCTTCCTG TCTGCGCCTT GCCACCGCTG</td>
<td>CGCGCCCTCTC TCAGCCCTCT GCCCGCGTTCT GCCACTGCGC</td>
</tr>
<tr>
<td>§START</td>
<td>§START</td>
<td>§START</td>
</tr>
<tr>
<td>Bovine</td>
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<td>Mouse</td>
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<tr>
<td>CTGGTCGAC CCGACGATG TCCGCAA</td>
<td>CCCG. AGC. C GACGCTAG TCCAGCAA</td>
<td>CTGGGCTCAC TGACAA GATG TCCAGCGGC</td>
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</tbody>
</table>
Figure 4. Ribonuclease protection assay. A, expected fragment size (bp) for each of AS mRNA 5’-UTRs relative to the RNA probe (138 bp for the 43-nt 5’-UTR; 161 bp for the 66-nt 5’-UTR; and 176 bp for the 92-nt 5’-UTR) and correspondence to the 5’-end of AS mRNA. B, autoradiograph of RPA fragments from 100 µg of endothelial cell total RNA (E) and 15 µg of liver total RNA (L) separated by a polyacrylamide sequencing gel. Molecular weight marker (bp markers) sizes are designated by base pair numbers (right).
Figure 5. Quantitation of the different forms of AS mRNA by real-time PCR. The percent of total AS mRNA for each of the 5’-UTR AS mRNA species (designated by 43 nt, 66 nt, and 92 nt, respectively) from endothelial cells (A) and bovine liver (B) as determined by real-time RT-PCR.
Appendix B (Continued)

**Figure 6. Efficiency of in vitro translation of the 5′-UTR AS mRNAs.** A, Western blot analysis of the translated protein. Full-length AS cDNA was subcloned to contain each of the AS mRNA 5′-UTRs as well as a control 5′-UTR (C) containing only a Kozak sequence (Kozak 1991) preceding the AUG start codon. These constructs, designated 92 nt, 66 nt, 43 nt, and control (C) were transcribed, capped (Ribo m7G Cap Analog), and translated. Biotinylated lysine was incorporated into the reaction for immunodetection with streptavidin horseradish peroxidase. Protein expression was quantitated by subtracting the negative control (no RNA added to the translation reaction) and normalizing to β-actin. B, expression level relative to the 43-nt 5′-UTR AS mRNA.
Appendix B (Continued)

Figure 7. Deletion and mutational analysis of the 5′-UTR AS mRNAs. Full-length AS cDNA deletion mutants were constructed to include 86, 81, 76, and 71 nt in the 5′-UTR. Constructs were also created in which the upstream uAUG was mutated to AAG (Mut1) or altered to a more favorable context (Kozak 1991) by changing nucleotides at -3 and +4 relative to the uAUG (GGG AUG CGC to AGG AUG GGC, Mut2). Constructs were transcribed, capped (Ribo m7G Cap Analog), and then translated. Biotinylated lysine was incorporated into the reaction for immunodetection with streptavidin horseradish peroxidase. Protein expression was quantitated by subtracting the negative control (no RNA added to the translation reaction) and normalizing to β-actin. Expression levels of the mutants are shown relative to the 43-nt 5′-UTR AS mRNA.
Figure 8. AS mRNA 5´-UTR effects on luciferase expression in endothelial cells.
BAEC were transiently transfected with the indicated AS mRNA 5´-UTR luciferase construct (Basic, pGL3Basic negative control; Control, pGL3Control positive control; and 43 nt, 66 nt, and 92 nt AS 5´-constructs, respectively). Luciferase activity was assayed 48 h after transfection, and results were normalized to renilla activity expressed from a co-transfected control vector. Error bars indicate the standard error of the mean from nine experiments.
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Review: The Caveolar Nitric Oxide Synthase/Arginine Regeneration System for NO Production in Endothelial Cells

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Summary

The enzyme endothelial nitric oxide synthase (eNOS) catalyzes the conversion of arginine, oxygen and NADPH to NO and citrulline. Previous results suggest an efficient, compartmentalized system for recycling of citrulline to arginine utilized for NO production. In support of this hypothesis, the recycling enzymes, argininosuccinate synthase (AS) and argininosuccinate lyase (AL), have been shown to colocalize with eNOS in caveolae, a subcompartment of the plasma membrane. Under unstimulated conditions, the degree of recycling is minimal. Upon stimulation of NO production by bradykinin, however, recycling is co-stimulated to the extent that more than 80% of the citrulline produced is recycled to arginine. These results suggest an efficient caveolar-localized arginine regeneration system, plays a key role in receptor-mediated stimulation of endothelial NO production. To investigate the molecular basis for the unique location and function of endothelial AS and AL, endothelial AS mRNA was compared with liver AS mRNA. No differences were found in the coding region of the mRNA species, but significant differences were found in the 5′-untranslated region (5′-UTR). The results of these studies suggest that sequence in the endothelial AS-encoding gene, represented by position –92 nt to –43 nt from the translation start site in the extended AS mRNA 5′-UTRs, plays an important role in differential and tissue-specific expression. Overall, a strong evidential case has been developed supporting the proposal that arginine availability, governed by a caveolar-localized arginine regeneration system, plays a key role in receptor-mediated endothelial NO production.

Key words: nitric oxide, eNOS, endothelial nitric oxide synthase, arginine, citrulline, arginine regeneration system, argininosuccinate synthase, argininosuccinate lyase, caveolae, nitric oxide production.
Introduction

Endothelial nitric oxide synthase (eNOS), the enzyme that catalyzes the production of NO from the amino acid arginine in endothelial cells, plays a key role in vasoregulation as well as in other important physiological processes such as angiogenesis. Impaired production of endothelial NO has been associated with hypertension, heart failure, hypercholesterolemia, atherosclerosis and diabetes (Govers et al. 2001; Maxwell 2002; Vallance et al. 2001). Circulating effectors, such as bradykinin, bind to receptors on the luminal surface of endothelial cells, signaling the transient release of NO to the adjacent smooth muscle layer and resulting in relaxation of the vessel wall.

The signal for eNOS activation is a transient increase in intracellular calcium, which activates the enzyme through binding of a calcium–calmodulin complex (Ca–Cam). Endothelial NOS activation also occurs in response to shear stress (Govers & Rabelink 2001; Maxwell 2002). Consistent with the important physiological roles of eNOS, the enzyme appears to be subject to multiple modes of regulation, in addition to primary regulation through reversible Ca–Cam binding and activation. These include reversible phosphorylation and palmitoylation, substrate and cofactor availability, dimerization of enzyme subunits, intracellular translocation and protein–protein interactions (Govers & Rabelink 2001). Several of these potential modes of regulation appear to be interrelated. As a component of caveolae, a subcompartment of the plasma membrane that serves to sequester proteins involved in cell signaling, eNOS may transiently interact with several different caveolar components. Previous work from several different laboratories has suggested that a diverse group of proteins, including calmodulin, caveolin-1, bradykinin B2 receptor, heat shock protein 90, argininosuccinate synthase (AS), argininosuccinate lyase (AL), Raf-1, Akt, extracellular signal-related kinase, eNOS interacting protein, eNOS traffic inducer and unidentified tyrosine-phosphorylated proteins (Govers & Rabelink 2001; Hellermann et al. 2000; Maxwell 2002; Nedvetsky et al. 2002), may be transiently and functionally associated with eNOS.

A potential limiting factor for endothelial NO production is the availability of the substrate, arginine. Intracellular levels of arginine have been estimated to range from 100
µmol l\(^{-1}\) to 800 µmol l\(^{-1}\), which is well above the \(K_m\) value of 5 µmol l\(^{-1}\) for eNOS (Harrison 1997). Endothelial NO production can, nonetheless, be stimulated by exogenous arginine (Vallance & Chan 2001). This phenomenon, termed the ‘arginine paradox’, suggests the existence of a separate pool of arginine directed to endothelial NO synthesis. As illustrated in Fig. 1, arginine has a number of metabolic roles in addition to NO production, including production of major metabolites such as urea, polyamines, creatine, ornithine and methylarginine derivatives. The observed stimulation of endothelial NO production by exogenous arginine suggests that the arginine directed to NO production may be segregated from bulk cellular arginine utilized for these other metabolic roles.

One possible site of control is at the level of arginine uptake. McDonald et al. (McDonald et al. 1997) showed that the CAT1 transporter, responsible for 60–80% of total carrier-mediated arginine transport into endothelial cells, colocalizes with eNOS in caveolae. They proposed that the arginine utilized by eNOS might, at least in part, be maintained by the CAT1 transporter. Another important mechanism for controlling the availability of arginine directed to NO production may be the regeneration of arginine from the other product of the eNOS-catalyzed reaction, citrulline. Hecker et al. (Hecker et al. 1990) initially demonstrated that citrulline, produced in the conversion of arginine to NO, can be recycled to arginine. A possible link between NO production and arginine regeneration from citrulline was subsequently established for other cell types (Nussler et al. 1994; Shuttleworth et al. 1995). This regeneration is catalyzed by the enzymes AS and AL, both of which also play an essential role in the urea cycle in liver. The potential importance of this regeneration system for endothelial NO production was supported by a report of two infants with a deficiency of AL who were shown to be hypertensive (Fakler et al. 1995). Upon infusion of arginine, the blood pressure of these infants decreased to near normal levels, suggesting a critical role for arginine regeneration in the regulation of systemic blood pressure. More recent evidence from DNA microarray analysis suggests an important role for the arginine regeneration system by clearly demonstrating significant and coordinate upregulation of AS-encoding gene expression in response to shear stress stimulation of endothelial NO production (McCormick et al. 2001). It was
concluded that available arginine is a prerequisite for NO production and that in the
absence of synthesis of additional eNOS, shear stress-induced increases in NO synthesis
depend on an increase in synthesis of arginine from citrulline through increased AS
expression. Although supplemental arginine can be beneficial in some cases (Wu et al.
2002), in other cases it may lead to adverse effects owing to the multiple metabolic roles
of arginine (Chen et al. 2003; Loscalzo 2003).

Recent work further supports the hypothesis that the arginine regeneration system,
comprised of a caveolar complex that includes eNOS, AS and AL, plays an important,
and most likely essential, role in the receptor-mediated production of NO by vascular
endothelial cells.

**Effects of exogenous arginine and citrulline on endothelial NO production**

Endothelial NOS is localized in plasmalemmal caveolae. The localization of
eNOS in this signaling subcompartment of the plasma membrane may have important
implications with regard to the regulation and catalytic efficiency of eNOS (Everson et al.
2001; Shaul 2002). We have recently found evidence for an efficient cycling of citrulline
to arginine, raising the possibility of a channeling complex of eNOS and the enzymes of
the citrulline–arginine cycle (AS and AL) localized in caveolae. Our initial research
effort that led to this finding was designed to test the hypothesis that an intracellular
pathway exists for the generation of methylarginines to regulate NO production in nitric
oxide-producing tissues. The goal of this initial work was to determine the physiological
significance of intracellular methylarginines as regulators of NOS activity. To examine
the levels of endogenous methylarginines, we developed methods that allowed for the
rapid and quantitative analysis (by HPLC) of arginine, citrulline and the methylarginines
from endothelial cell extracts. There was no apparent change in levels of methylarginines
following stimulation of endothelial cells with either bradykinin or the calcium ionophore
A23187. In an attempt to raise intracellular methylarginine levels, and further test our
hypothesis, we added citrulline, which we expected to inhibit dimethylarginine
dimethylaminohydrolase, the enzyme that converts $N^G$-methylarginine or $N^G,N^G$-
dimethylarginine to citrulline and monomethylamine or dimethylamine, respectively. The
objective was to determine whether inhibition of the degradation of methylarginines
would increase their intracellular concentrations and thereby inhibit NO production. To our surprise, stimulation of NO production by bradykinin was increased by the addition of citrulline, rather than decreased, and there was no apparent change in methylarginine levels. To further examine the molecular basis for the stimulation of NO production by citrulline, we compared the effect of exogenous citrulline with the effect of exogenous arginine on NO production and levels of intracellular arginine following bradykinin activation. Surprisingly, added arginine did not cause as great an increase in endothelial NO production as did added citrulline. In addition, there was a much larger increase in intracellular arginine in response to exogenous arginine compared with exogenous citrulline. Added citrulline caused only a modest increase in intracellular arginine, while added arginine caused a substantial increase. Thus, there appeared to be no correlation between total intracellular arginine levels and endothelial NO production. To the best of our knowledge, this represents the first attempt to correlate NO production with the levels of intracellular arginine. Furthermore, the effects of arginine and citrulline on NO production appeared to be synergistic, since a combination of arginine and citrulline stimulated endothelial NO production more than did either arginine or citrulline alone (Flam et al. 2001). Since arginine has a number of potential metabolic fates, while citrulline has only one known metabolic fate (Fig. 1), the efficiency of NO production could be enhanced if a separate pool of arginine is maintained by endothelial cells. Recycling the product of the NOS-catalyzed reaction, citrulline, back to arginine via the enzymes of the arginine regeneration system, AS and AL, would maintain this separate pool. The pool of arginine used for NO synthesis would be essentially isolated from the bulk of intracellular arginine through the efficient operation of an arginine regeneration system. The apparent efficiency of the process suggests a channeling of intermediates and a compartmentalized complex of eNOS and enzymes of the arginine regeneration system. These results further support a model in which eNOS is localized together with this arginine regenerating system, and regulatory components, to ensure optimal efficiency of NO production and regulation without affecting other arginine-dependent cellular processes.
Caveolar localization of arginine regeneration enzymes with eNOS

Endothelial NOS is targeted by acylation to caveolae, where it interacts with caveolin-1 (Everson & Smart 2001; Shaul 2002). In liver cells, the arginine-generating enzymes AS and AL are associated with the outer mitochondrial membrane, reflecting the functional role of these enzymes in the production of urea (Cohen et al. 1996). To test the model for a colocalization of AS and AL with eNOS, we used two different fractionation protocols for the purification of caveolae (Smart et al. 1995; Song et al. 1996). Both protocols generated a caveolar membrane fraction that was highly enriched in caveolin-1, eNOS, AS and AL (Flam et al. 2001). These results support the proposal that a separate pool of arginine, directed to NO synthesis, is effectively separated from the bulk of intracellular arginine through the functional localization of arginine regeneration enzymes and eNOS with plasmalemmal caveolae. A possible consequence of this functional association would be the channeling of intermediates through AS, AL and eNOS such that intermediates of the complex would not equilibrate with bulk intracellular arginine.

Degree of recycling

Cellular activity of eNOS has been estimated by measuring the rate of conversion of $[^3]$Harginine to $[^3]$Hcitrulline (Hardy et al. 2002). If recycling of citrulline to arginine is tightly coupled to NO production, this measurement would underestimate the cellular activity of eNOS. Estimating cellular activity of eNOS by measuring rate of production of NO (as the degradation product nitrite), on the other hand, should give a better estimate of cellular activity of eNOS. To test this hypothesis, and to estimate the degree of recycling of citrulline to arginine, we simultaneously measured the apparent rate of arginine-to-citrulline conversion and the rate of production of NO under both unstimulated and stimulated (addition of bradykinin) conditions. The ratio of these activities was close to one under unstimulated conditions. An increase in the ratio of NO produced to citrulline produced was approximately eight upon exposure of endothelial cells to agonist (B. R. Flam, D. C. Eichler and L. P. Solomonson, unpublished), indicating that recycling and NO production were costimulated. These preliminary results suggest an efficient caveolar complex for the regeneration of arginine directed to
Appendix C (Continued)

receptor-mediated production of NO in endothelial cells and an efficiency of greater than 80% for the recycling of citrulline to arginine under conditions of maximum stimulation of NO production. Although recycling of citrulline to arginine has been assumed to be important for conservation and efficient utilization of arginine, the degree of recycling relative to NO production has not, to the best of our knowledge, been quantified. Our results suggest that this recycling, especially under stimulated conditions, may play a more important role in endothelial NO production than previously recognized.

**Molecular basis for functional role and location of endothelial AS**

In liver tissue, AS plays an essential role in urea synthesis and appears to be associated with the outer mitochondrial membrane (Cohen & Kuda 1996). By contrast, endothelial AS appears to be the rate-limiting enzyme in the recycling of citrulline to arginine used for NO synthesis and is localized in caveolae (Flam et al. 2001). Immunoblotting experiments suggested small differences in subunit molecular masses and isoelectric points of endothelial AS compared with liver AS (B. R. Flam, D. C. Eichler and L. P. Solomonson, unpublished). We speculated that these differences could be due to a splice variant, but analysis of the coding sequence of AS mRNA indicated no differences between the mRNA from endothelial cells and liver (Pendleton et al. 2002). Because upstream and downstream untranslated regions (UTRs) of mRNA can influence regulation of gene expression, we carried out both 5′-RACE (rapid amplification of cDNA ends) and 3′-RACE analysis to investigate possible differences in the UTRs. We found AS mRNA species with three different length 5′-UTRs in endothelial cells (Fig. 2). Only one of these products, the shortest 5′-UTR of 43 nt, was quantitatively expressed in liver. No significant variation was found in the 3′-UTR. The 5′-RACE analysis identified endothelial AS mRNA species with extended 5′-UTRs of 66 nt and 92 nt, in addition to a major 43 nt 5′-UTR AS mRNA (Fig. 2). Compositional analysis revealed that all three AS mRNA 5′-UTRs were enriched in G+C content (approximately 76%) and were likely to form complex and stable secondary structures. An upstream open reading frame (uORF) that was out-of-frame with the AS mRNA AUG start codon was detected in the 66 nt and 92 nt 5′-UTRs. RNase protection analysis (RPA) and real-time reverse transcriptase-PCR (RT-PCR) verified and quantified the differential expression of the
extended 5′-UTR species relative to the major 43 nt 5′-UTR AS mRNA. Estimates from RPA of the amount of the 92 nt and 66 nt species, relative to the 43 nt species, were approximately 15% and 13%, respectively.

Features of mRNA UTRs, specifically uORFs, are regarded as important determinants of translational efficiency and may have important biological implications for the regulation of translation. We therefore designed experiments to determine to what extent the various 5′-UTRs of AS mRNA influenced translation. Translational efficiencies for the 66 nt and 92 nt AS 5′-UTR constructs were 70% and 25%, respectively, of the translational efficiency for the 43 nt 5′-UTR AS mRNA. Sequential deletions, starting with the 5′-terminus of the 92 nt 5′-UTR construct, resulted in a corresponding increase in translational efficiency, but the most pronounced effect resulted from mutation of the uORF, which restored translational efficiency to that observed with the 43 nt species. When the different AS mRNA 5′-UTRs, cloned in front of a luciferase reporter gene, were transfected into endothelial cells, the pattern of luciferase expression was nearly identical to that observed for the different 5′-UTR AS mRNAs in endothelial cells. These results suggest that a complex transcriptional/translational infrastructure exists to coordinate AS expression and NO production (Pendleton et al. 2002).

Model for coupling of arginine regeneration to endothelial NO production

A model depicting our view of the coupling of arginine regeneration to endothelial NO production through the compartmentalized complex of AS, AL and eNOS is shown in Fig. 3. This coupling may be largely ‘disengaged’ under unstimulated conditions but is ‘engaged’ and tightly coupled in response to agonists such as bradykinin. The molecular determinants and mechanisms involved in this coupling are not fully understood at this time. Based on our studies, and evidence from other labs, we believe the coupling of arginine regeneration to endothelial NO production is important for the overall regulation of endothelial NO production and may be essential for agonist-stimulated endothelium-dependent vasorelaxation.
Acknowledgements

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Figure 1. Metabolic roles and fates of arginine. In addition to incorporation into protein, arginine serves as a metabolic precursor for several important metabolites, as indicated by the arrows. Also indicated is the two-step conversion of citrulline to arginine.
Figure 2. Novel 5' untranslated regions (UTRs) of endothelial argininosuccinate synthase mRNA.

92 nt 5’-UTR

\[\text{CG CUG CCC CCC GGC CCC GAG CUU AUA ACC CGG GAU GCG CGC CGA AAC}\]

66 nt 5’-UTR

\[\text{START}\]

43 nt 5’-UTR

\[\text{CGG CCC UGC UCC GCC GAC UGC UGC CGC CGC UGG UCA CCC GUC ACG AUG UCC GGC ...}\]
Figure 3. Model for the coupling of endothelial NO production to the regeneration of the substrate, arginine, from the product, citrulline. Shown is the CAT1 transporter involved in arginine transport and the complex of argininosuccinate synthase and argininosuccinate lyase with endothelial nitric oxide synthase.
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Appendix D

Structures of Commonly Used Compounds

Chemical Structures

Sugar
D-(-)-Glucosamine hydrochloride

Post-translational modification
Proteins are glycosylated on serine or threonine residue with a monosaccharide β-N-acetylglucosamine, in an O-glycosidic linkage (O-GlcNAc)
Appendix D (Continued)

Inhibitors

Nitric oxide synthase (NOS) inhibitor
\( \text{N} \omega-\text{Nitro-L-arginine methyl ester hydrochloride (L-NAME)} \)

L-glutamine:D-fructose-6-phosphate amidotransferase (GFAT) inhibitor
6-Diazo-5-oxo-L-norleucine (DON)

\( \beta \)-D-N-acetylglucosaminidase (O-GlcNAcase) inhibitor
O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-N-phenylcarbamate (PUGNAc)
About the Author

Brenda R. Flam was born in New Jersey and attended the University of Rochester, Rochester, NY where she earned a B.S. degree in Chemical Engineering. She worked at Princeton University in the Ocean Tracers Laboratory studying ocean currents and then worked as a research biochemist studying compounds to combat the side effects of type 2 diabetes at Wyeth in Princeton, NJ. She then moved to the Institute for Diabetes Discovery in Branford, CT where she developed and modified in vitro assays for high throughput screening for type 2 diabetic compounds. Brenda entered the doctoral program in Medical Sciences in the Department of Molecular Medicine, School of Basic Biomedical Sciences, College of Medicine, at the University of South Florida to pursue additional scientific training.