

**NITROGEN METABOLISM: ENZYME EXPRESSION AND PROTEIN  
INTERACTIONS IN THE UREA AND NITRIC OXIDE CYCLES**

By

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Dissertation

Submitted to the Faculty of the  
Graduate School of Vanderbilt University  
in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Human Genetics

May, 2010

Nashville, TN

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This dissertation is lovingly dedicated to my amazing mother and father, Heather and Ray Neill, for their infinite encouragement and without whose caring support this work would not have been possible.

## ACKNOWLEDGEMENTS

This work would not have been possible without the Program in Human Genetics at Vanderbilt University providing the training and program support for my education. My thesis committee has been influential in guiding and mentoring me throughout my time here at Vanderbilt. I would like to thank Dr. Michael Aschner, Dr. Douglas Mortlock, Dr. Deborah Murdock, Dr. Marshall Summar and Dr. Tony Weil for taking the time to help me and challenge me in my development and growth as a research scientist. I would particularly like to thank Dr. Deborah Murdock for her unyielding optimism and for her encouragement and support in developing a hypothesis-driven dissertation. Thank you for believing in me as a scientist. I would like to thank Dr. Marshall Summar, my mentor, for giving me a position in the lab, for encouraging me to develop my own research project and for inspiring me to make connections between my research and clinical medicine. I would like to thank the Summar lab members, especially Gary Cunningham and our collaborators Dr. Judy Aschner and Heng Zeng for their support.

I would like to thank the professors, scientists, friends and family who have supported me throughout my career and have truly made this work possible for me to achieve. Dr. Robert Gatenby and Dr. Robert Gillies – thank you for recognizing my passion for scientific research and inspiring me to attend graduate school. I would like to thank my undergraduate mentor at the University of Arizona, Terry Thompson, for teaching me to believe in myself, for encouraging me to never set

limits on my abilities or accomplishments and for inspiring me to maintain a close connection with the community through service.

To the friends that I met through IGP and the Human Genetics Program, especially Dr. Rebecca Zuvich and Lauren Walters, thank you for always being there to listen and support me and for giving me the strength I needed to finish this dissertation. Together we have embodied the message that a woman can be intelligent, sociable and fashionable; we have such exciting futures ahead of us –I know we will be life-long friends and I cannot wait to see where our bright futures will lead.

To my brother and nephew, Matthew and Nolan, thank you for your unending love and support. To my parents: thank you for teaching me the value of hard work, integrity and character. Thank you for always standing by me and reminding me that I can accomplish whatever I set my mind to. Mom: thank you for the limitless ways you have always supported my dreams and believed in me, especially when I didn't believe in myself. Dad: thank you for showing me the world and sharing with me your love for travel, adventures and culture - most importantly thank you for always being there at every milestone in my life. Most importantly thank you for giving me every opportunity in the world and for making the many sacrifices that have helped make my dreams come true.

To my true love Jordan - you have changed my life in ways I never thought were possible. You gave me the courage to follow through and never give up. Thank you for always standing by me and for making me smile and laugh each and every day. I love you.

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## LIST OF ABBREVIATIONS

cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
DNA	deoxyribonucleic acid
mRNA	messenger ribonucleic acid
NO	nitric oxide
NOS	nitric oxide synthase
NO <sub>x</sub>	nitric oxide metabolites
PCR	polymerase chain reaction
UCD	urea cycle disorder
qRT-PCR	quantitative polymerase chain reaction
SNP	single nucleotide polymorphism
TAP	tandem affinity purification

### Gene Abbreviations

ASS	argininosuccinate synthase
ASL	argininosuccinate lyase
ARG1	arginase I
ARG2	arginase II
β-actin	beta-actin
Citrin	citrin
CPSI	carbamoyl phosphate synthase
eNOS	endothelial nitric oxide
GAPDH	glyceraldehyde phosphate dehydrogenase
iNOS	inducible nitric oxide
nNOS	neuronal nitric oxide
NAGs	n-acetyl glutamate synthase
OTC	ornithine transcarbamoylase
ORTN1	SLC 2515

# CHAPTER I

## OVERVIEW

There are approximately 20,000 – 25,000 protein coding genes in the human body. These genes code for a variety of proteins such as structural proteins, ligand-binding proteins, cell signaling proteins and enzymes. Enzymes catalyze important reactions that include producing energy via the Krebs cycle, oxidizing fatty acids via  $\beta$ -oxidation and detoxifying the body of nitrogen via the urea cycle. The focus of this dissertation involves nitrogen metabolism and how enzyme expression and regulation regulates their function. Research in this field has typically focused on the regulation of individual enzymes and the effects of single nucleotide polymorphisms (SNPs) in each gene on nitrogen metabolism. This dissertation aims to consider the metabolic system, at the mRNA and protein level. The effect of substrates and products in the cellular milieu on enzyme function as well as potential interactions between enzymes is also considered. The molecular regulation and potential interactions between enzymes remains poorly understood in the field of urea cycle and nitrogen metabolism. Specifically we hypothesize that the enzymes of the urea and nitric oxide cycle are regulated in a coordinated and tissue-specific manner and that these enzymes interact in multi-protein complexes whose components differ depending on cell type. The aims of our project are to determine where the enzymes of the urea and nitric oxide cycles are expressed, to determine whether expression of these enzymes changes in response to pathway-specific stimuli and finally, to

determine whether enzymes of the urea and nitric oxide cycles interact at the protein level.

The introduction to nitrogen metabolism is presented in Chapter II. Additionally, our hypothesis and specific aims of this dissertation are presented in this Chapter.

To expand on clinical studies which have shown that citrulline can induce nitric oxide production, in Chapter III we examined the enzymes involved in this pathway using an *in vivo* mouse model. This study revealed that the concentration of nitric oxide metabolites correlates with the concentration of citrulline in mouse tissues.

To gain a better understanding of where these enzymes are expressed in normal human tissue, we studied mRNA expression utilizing quantitative real-time PCR (qRT-PCR) in Chapter IV. These studies indicate that there is overlap in the expression between the urea cycle specific genes, the NO cycle specific genes (primarily the NO synthases) and the shared genes (argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL)).

Chapter V considers the effects of intermediates and urea and/or nitric oxide specific stimuli on the expression of enzyme mRNA, protein as well as levels of pathway intermediates (arginine and citrulline) and nitric oxide metabolites. These studies indicate a cell-type specific response in the mRNA and protein expression of urea and nitric oxide cycle enzymes. Additionally, the concentrations of pathway intermediates (arginine and citrulline) as well as the end-product (nitric oxide metabolites) can be affected by stimuli in the extracellular environment.



In Chapter VI, our focus was to identify putative interactions between ASS, ASL and their protein counterparts. In this Chapter, experiments demonstrated that ASS and endothelial nitric oxide (eNOS) co-localize in primary piglet pulmonary endothelial cells and that ASS and ASL exist in a larger complex with several other proteins.

Chapter VII provides a summary of the content of this dissertation and describes future experiments which will be important in elucidating the mechanism of protein-protein interactions as a potential form of enzyme regulation in nitrogen metabolism and to identify the specific protein components that interact with ASS and ASL.

## CHAPTER II

### INTRODUCTION

One of the first scientists to draw connections among the fields of genetics, molecular biology and biochemistry was Sir Archibald Garrod (1857-1936). Through his experimental work on alkaptonuria also known as black urine disease, Sir Garrod proposed a link between the inability to make a particular enzyme and an inherited disease (Garrod, 1996), alluding to the idea that genes' products play important roles in biochemistry. An insightful remark stated by Garrod, "I believe that no two people are alike chemically more than structurally" underscores how variability in DNA may be reflected in metabolic capabilities. Subsequent work demonstrated errors in metabolism, such as alkaptonuria, are often inherited in a recessive manner and are due to the absence of a critical enzyme in a metabolic pathway (Bearn, 1994), underscoring the importance of genetics in metabolism.

The work of Garrod was one of the first examples connecting a physical syndrome to a specific metabolic pathway, a series of chemical reactions occurring within a cell. Metabolism is defined as "the complex of physical and chemical processes involved in the maintenance of life" (Schilling, Letscher, and Palsson, 2000). Metabolism encompasses a wide range of chemical reactions and transport mechanisms that are required to support life. Metabolic objectives or fates are achieved through the complex regulation of enzyme expression from the transcriptional level through post-translational, including physical interactions

between enzymes and co-factors. By controlling enzyme activity, cells are able to direct the production and distribution of specific metabolites; ultimately allowing different cells, tissues and organs to achieve different metabolic fates. Moreover, many metabolic pathways overlap to form metabolic networks, adding additional layers of complexity. It is critical that researchers studying metabolic pathways consider the broader connections between these pathways rather than study individual enzymes or a specific metabolic pathway in isolation. Instead, scientists must consider the fates of substrates and end-products, in addition to the interconnections between metabolic pathways, in order to fully characterize metabolic networks and understand the diseases and disorders which result from errors in these pathways.

### **Protein-Protein Interactions**

The interaction between proteins in biochemical processing is seen in all organisms. The interaction between proteins plays a fundamental role in many biological functions such as the regulation of metabolic pathways, the cell cycle, DNA replication and protein synthesis (Jones and Thornton, 1996). Complexes of proteins are found in every cellular location, including the cell organelles, the cytosol and the cell membranes (Hardy, Holmgren, Johansson, Sanchez, and Hirst, 1988). These complexes are biologically important as they mediate biochemical phenomena such as enzyme cooperation and signal transduction (Jones *et al.*, 1996). The importance of protein interactions in metabolic systems is reflected in the quantity of research that is devoted to discovering novel protein associations and the continued

development of novel techniques to advance the discovery of protein interactions. Studying protein-protein interactions is essential to define the networks that contribute to maintain homeostasis of an organism's body functions (Sam, Liu, Li, Friedman, and Lussier, 2007). Proteins are engaged in a complex array of physical and functional interactions with other proteins and macromolecules under physiological conditions. Such interactions are regulated both spatially and temporally within a cell to mediate cellular processes, including biochemical pathways and metabolism. The identification and characterization of the components involved in these complexes and understanding how they interact to carry out their biological functions is important for understanding metabolic and biological processes at the molecular level and for targeting strategies for manipulating them.

The organization of metabolic enzymes that catalyze sequential biosynthetic steps into multi-component complexes are an important example of how proteins interact on the molecular level. Protein interactions provide an important level of regulation, because instead of proteins and substrates colliding in a diffusion-dependent manner, proteins generally interact with each other and form larger assemblies in a time- and space-dependent manner (Alberts, 1998; Bauer and Kuster, 2003). Protein interactions may exert their effects at the metabolic level in a variety of ways. These interactions may alter the kinetic properties of enzymes through the altered binding of substrates, the formation of a novel binding site or by changing the specificity of a protein for its substrate (Phizicky and Fields, 1995). Additionally protein interactions may be a mechanism which permits substrate channeling.

Tryptophan synthetase enzyme in *Neurospora crassa* is a model for protein interactions and substrate channeling. The complex consists of two subunits, each of which is required for the formation of tryptophan. These two subunits form a channel through which the substrate indole is transported to form tryptophan (Hyde and Miles, 1990). Because these interactions are often essential to support the metabolic activities of the cell and maintain homeostasis, disruption of these complexes may have drastic clinical implications.

The classic model of molecular disease is the effect of a genetic change on the individual protein and its isolated function; however, disruption of protein interactions can also result in diseases such as hemophilia, neurodegenerative diseases and metabolic diseases (Li and Li, 2004; Pipe, Saenko, Eickhorst, Kembell-Cook, and Kaufman, 2001). Additionally, protein-protein interactions have also become a target for drug discovery (Dev, 2004; Fuentes, Oyarzabal, and Rojas, 2009; Gerrard, Hutton, and Perugini, 2007). One of the goals of genomic, proteomic and biochemical research is to detect and discover all possible protein interactions in metabolic pathways and determine how these interactions are important in disease. More research is required to bridge the existing gap and more clearly define the relationships between protein interactions and disease. Important insights may be gained from studying protein interactions, particularly in the area of metabolism.

## Metabolic Complexes

The metabolic activities of *Homo sapiens* are optimized and highly coordinated. At the cellular level, channeling of substrates to their target enzymes is facilitated by the compartmentation of the cell into different organelles. Even though most enzymes are soluble proteins, they do not spread freely in the cytoplasm as a “bag of enzymes” (Mathews, 1993). Enzymes that catalyze sequential reactions are co-localized within cells and bound to structural elements of cells (Srere, 1987). This co-localization provides important benefits for the metabolic pathway and the organism. These benefits include: protecting unstable or scarce metabolites, maintaining concentration gradients and protecting the solvation capacity of cell water, affording kinetic advantages for enzymes (Mathews, 1993). Srere coined the term “metabolon” to denote a complex of sequential metabolic enzymes, which may involve both transiently associated enzymes and a multifunctional enzyme complex in which the enzymes form tighter bonds. An important biological example of a metabolon is the formation of the pyruvate dehydrogenase complex (PDC) (Leong, Brownsey, Kulpa, and Allard, 2003; Reed LJ, 1974; Srere, 1987).

The organization of cooperating enzymes into macromolecular complexes is a central feature of metabolism. For example, the pyruvate dehydrogenase complex (PDC), which catalyzes the conversion of pyruvate to acetyl CoA for energy generation in the carbohydrate metabolism pathway, is one of the largest and most complex multi-enzyme systems (Figure 2.1) (Milne *et al.*, 2002; Patel and Roche, 1990). In mammalian cells, the PDC is found in the matrix space of the

mitochondria (Addink, Boer, Wakabayashi, and Green, 1972).

The PDC complex contains a core consisting of the dihydrolipoyl dehydrogenase enzyme and two regulatory enzymes – a kinase and a phosphatase (Reed LJ, 1974).

Electron microscopy studies have shown these enzymes to associate as a macromolecular complex and that

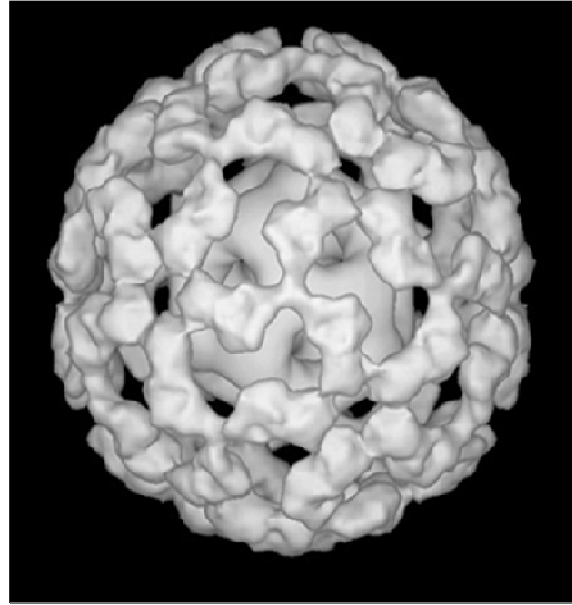


Figure 2.1 Stereo Representation of the PDC Complex.

From (Milne, Shi, Rosenthal, Sunshine, Domingo, Wu, Brooks, Perham, Henderson, and Subramaniam, 2002)

protein association is necessary for the overall reaction to proceed. For example, in mouse models where the alpha subunit of PDC was knocked out, mice fed a high fat diet had left ventricular hypertrophy and reduced systolic function compared to wild-type mice (Sidhu, Gangasani, Korotchkina, Suzuki, Fallavollita, Canty, Jr., and Patel, 2008), demonstrating an important physiological effect when the protein complex was disrupted. The PDC complex thus provides an example of how a specific interaction gives rise to a level of complexity that must be accounted for when a scientist wishes to understand metabolic regulation and metabolic disorders.

Although there are many well-characterized examples of metabolons, including PDC, enzymes involved in the urea and nitric oxide cycles remain poorly examined in terms of protein interactions (Velot, Mixon, Teige, and Sreer, 1997; Winkel, 2004). The urea cycle is a particularly interesting potential model of a

metabolon because the enzymes involved in this pathway are distributed in two different compartments, the mitochondria and the cytoplasm (Cheung, Cohen, and Rajjman, 1989). This becomes more interesting when one considers that two enzymes of the urea cycle (argininosuccinate synthase and argininosuccinate lyase) are shared key components of the nitric oxide production cycle. The ultimate goal of studying the interactions between proteins is to understand the consequences of the interaction for metabolic function and disease. Studying protein interactions in the urea and nitric oxide cycles may help elucidate the role of metabolons in nitrogen metabolism.

### **The Urea Cycle**

The urea cycle is the primary mechanism of nitrogen metabolism in mammals. The cycle is a complex series of biochemical reactions that produces urea from ammonia (Figure 2.2) (Scaglia, Brunetti-Pierri, Kleppe, Marini, Carter, Garlick, Jahoor, O'Brien, and Lee, 2004) and is highly conserved in all mammalian species (Camacho, Obie, Biery, Goodman, Hu, Almashanu, Steel, Casey, Lambert, Mitchell, and Valle, 1999; Morizono, Caldovic, Shi, and Tuchman, 2004). The complete urea cycle requires the activity of six enzymes: carbamoyl phosphate synthetase (CPSI), *n*-acetyl glutamate synthase (NAGs), ornithine transcarbamoylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL), arginase I (ARG1) and two transporters, citrin and ORTN1 (SLC25A15), all of which are encoded by single genes.



The urea cycle is the only known metabolic pathway capable of converting nitrogen derived from protein intake and/or protein degradation into urea. Although several tissues express some urea cycle enzymes, only the liver has the full metabolic capacity of detoxifying ammonia to urea. The biochemical reactions of the urea cycle also produce three physiologically important metabolites: ornithine, citrulline, and arginine. These metabolites are the substrates of a number of physiological biochemical reactions including new protein production, polyamine production, and the creation of nitric oxide through the nitric oxide cycle.

In the cell, the urea cycle is compartmentally divided by the mitochondria into segments. The proximal or mitochondrial urea cycle enzymes are CPSI, NAGs and OTC. The distal or cytoplasmic urea cycle enzymes are ASS, ASL and ARG1. The mitochondrial membrane transport of citrulline and ornithine connects the mitochondrial and cytoplasmic portions of the urea cycle together. Over the past few decades, evidence has been presented for metabolite channeling in both the mitochondrial and cytoplasmic parts of the urea cycle (Cheung *et al.*, 1989;Cohen, Cheung, and Rajjman, 1987;Powers-Lee, Mastico, and Bendayan, 1987). However, data directly demonstrating interactions between the enzymes of this pathway are still lacking.

Forming a urea cycle metabolon may have several advantages. Because the urea cycle generates intermediates that are used for other biochemical pathways, one advantage may be to shield these substrates from competing pathways. For example, the urea cycle generates the intermediate arginine, which is used in the nitric oxide

pathway to form nitric oxide, translation to make protein, and the urea cycle to generate urea. Physically protecting arginine for nitric oxide production may increase the chances of a sustained production of nitric oxide. This concept leads to the idea of available and unavailable substrates and intermediates. In the urea cycle and nitric oxide cycle, citrulline would be an available intermediate since it is exported from the mitochondria to enter either pathway. Complexes may also incorporate components which compete with each other as a form of regulation. Recent data shows that arginase is often expressed in cells producing nitric oxide and is thought to compete for arginine with NOS as a regulatory mechanism (Scalera, Closs, Flick, Martens-Lobenhoffer, Boissel, Lendeckel, Heimburg, and Bode-Boger, 2009). The study of its interaction with NO producing complexes will provide insights into the critical regulation of NO production.

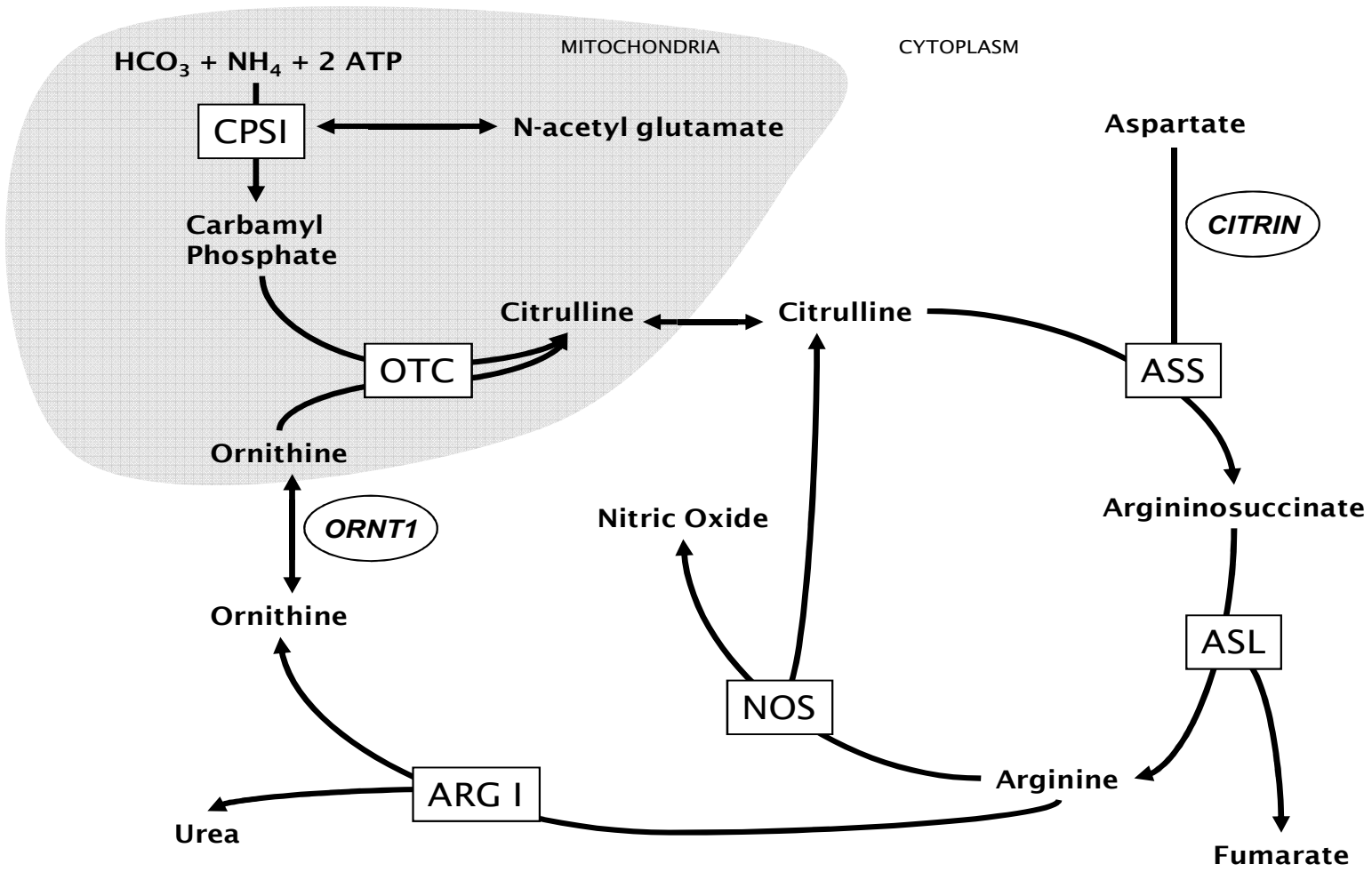


Figure 2.2 Schematic Representation of the Urea and Nitric Oxide Cycles.

## **The Nitric Oxide Cycle**

The urea cycle and the nitric oxide (NO) cycle are linked in that these two pathways share the use of two enzymes, argininosuccinic acid synthase (ASS) and argininosuccinic acid lyase (ASL) and a transporter, citrin (Figure 2.4). This represents a biologic frugality by having single genes participate in biochemically distinct processes as component pieces. The complete nitric oxide cycle involves the activity of the enzymes ASS and ASL as well as one of the three nitric oxide synthases, endothelial (eNOS), inducible (iNOS), and neuronal (nNOS). Additionally, the nitric oxide cycle uses the transporter citrin to import aspartate which condenses with citrulline (generated by the mitochondrial portion of the urea cycle or by the cleavage of arginine by NOS) to produce fumarate, which returns to the Krebs cycle.

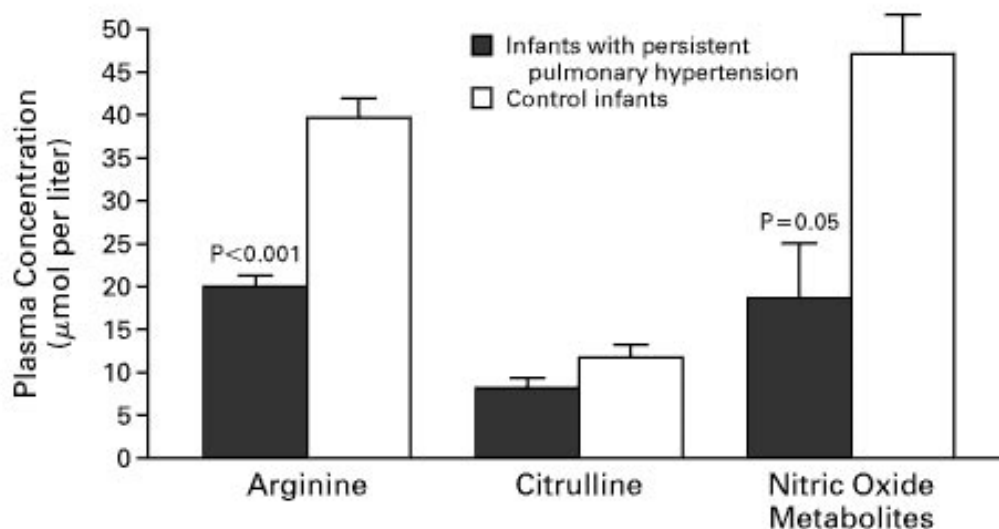
Nitric oxide is a vasodilator and is synthesized by ASS, ASL and one of three different nitric oxide synthases: eNOS, iNOS and nNOS. Nitric oxide production is tightly regulated at both the transcriptional and post-translational level (Kelm, 2003; Kelm and Schrader, 1990; Kobayashi, Sinasac, Iijima, Boright, Begum, Lee, Yasuda, Ikeda, Hirano, Terazono, Crackower, Kondo, Tsui, Scherer, and Saheki, 1999; Mori and Gotoh, 2004). A separate gene encodes each NOS isoform and each isoform has a distinct but overlapping tissue distribution. For example, nNOS is mainly expressed in neurons, but is also expressed in other tissues, including skeletal muscle, iNOS has low basal expression levels and is usually only identifiable in the presence of cytokines or other stimuli, and eNOS is the predominant isoform in vascular endothelial cells, but it is also expressed in some other tissues. It is not

known why three isoforms of NOS are needed since they conduct biochemically identical processes (Kone, 2001).

NO plays an important role in many tissues such as the cardiovascular system, the immune system, and the vascular endothelium and NO has many roles including neurotransmission, vasodilation, and macrophage bactericidal and tumoricidal activities (Ignarro, Cirino, Casini, and Napoli, 1999). Endogenous eNOS production is one of the most important modulators of vascular tone; and numerous studies have shown that it may protect against the development of pulmonary hypertension and other vascular diseases (Ekelund and Mellander, 1996;Kelm, 2003;Kelm, Feelisch, Deussen, Strauer, and Schrader, 1991;Kelm *et al.*, 1990;Koizumi, Gupta, Banerjee, and Newman, 1994;Mellander, Bjornberg, Ekelund, and Alm, 1997;Mellander and Ekelund, 1995). In the endothelium, eNOS produces vasodilation by the activation of soluble guanylate cyclase, the production of cyclic guanosine monophosphate (cGMP) and the activation of downstream effectors. The bioavailability of NO in the endothelium is dependent on the expression of eNOS, which is itself dependent on citrulline generated by the enzymes ASS and ASL, demonstrating the important connections between the urea and NO cycles.

## **The Clinical Connection between the Urea Cycle Enzymes and Nitric Oxide Production**

It has been shown clinically that adequate expression and activity of urea cycle enzymes is critical for endogenous nitric oxide production. Single nucleotide polymorphisms (SNPs) within the first gene of the urea cycle, carbamoyl phosphate synthetase I (CPSI), are associated with low circulating concentrations of two urea cycle intermediates citrulline and L-arginine, and impaired NO production in a host of disease pathologies including vascular diseases (Miller, Tang, Keech, Pigott, Beller, and Celermajer, 2000; Pearson, Dawling, Walsh, Haines, Christman, Bazyk, Scott, and Summar, 2001; Scaglia *et al.*, 2004; Summar, Gainer, Pretorius, Malave, Harris, Hall, Weisberg, Vaughan, Christman, and Brown, 2004). Persistent pulmonary hypertension (PPHN) of the newborn is a cardiopulmonary disorder characterized by hypoxia, cyanosis and respiratory distress. This common condition affects approximately 1 in 500 births (Latini, Del, De, Verrotti, and Bossone, 2008). Researchers discovered that infants who had PPHN had lower levels of citrulline, arginine and nitric oxide metabolites compared with infants who did not have PPHN (Table 2.1) (Pearson *et al.*, 2001). Additionally, it has been reported that the synthesis of arginine is affected by a functional SNP (T1405N) in the CPSI gene, the rate-limiting gene of the urea cycle (Pearson *et al.*, 2001). It is possible that there may be a genetically predetermined capacity of this enzyme can dictate one's susceptibility to PPHN. These experiments highlight the need to determine the molecular basis of clinical phenomena.



**Figure 2.3** Infants with PPHN have less circulating arginine, citrulline and nitric oxide metabolites than control infants. From: (Pearson *et al.*, 2001)

Additionally, a study reported that systemic hypertension, a consequence of impaired nitric oxide function, in two newborn patients that were lacking the ASL enzyme (Brunetti-Pierri, Erez, Shchelochkov, Craigen, and Lee, 2009). L-Arginine and/or citrulline administration increases NO production by providing increased substrate supply for the pathway and has been beneficial in treating patients with vascular diseases (Miller *et al.*, 2000; Pearson *et al.*, 2001; Summar *et al.*, 2004). However, L-arginine therapy is hindered by the fact that arginine is rapidly degraded by intestinal arginase activity and we hypothesize that if the urea cycle cytoplasmic enzymes exist in a functional complex, the enzymes may not be able to access arginine. Conversely, citrulline is readily absorbed by the body, and cells are able to convert it to arginine using ASS and ASL to L-arginine, augmenting NO-dependent signaling (Schwedhelm, Maas, Freese, Jung, Lukacs, Jambrecina, Spickler, Schulze,

and Boger, 2008). We hypothesize that citrulline is the preferential substrate which gains entry to the metabolic complex of urea cycle enzymes to produce nitric oxide. Alterations in the urea cycle that change the concentration of the intermediates citrulline and arginine can therefore affect the production of NO, which is harmful in excess or deficit (Brusilow SW and Horwich AL, 2001).

### **Evidence for Protein Interactions in the Urea and Nitric Oxide Cycles**

Over the past several decades, evidence has been presented which supports the concept of metabolite channeling in the urea cycle. Immunocytochemical analysis determined that the first two enzymes of the urea cycle, CPSI and OTC, are localized to the inner membrane of the mitochondria (Powers-Lee *et al.*, 1987). Studies conducted by Cohen *et al.* demonstrated that ornithine is channeled to the OTC enzyme (Cohen *et al.*, 1987). Because CPSI and OTC have the ability to interact with the inner mitochondrial matrix, we hypothesize that interactions might occur between the cytoplasmic enzymes of the urea cycle as well. Evidence that citrulline exported from the mitochondria is channeled for urea formation in the cytosol from the work of Cohen and colleagues supports the potential for protein interactions in this cellular compartment.

Evidence suggests the urea cycle may be organized into a “metabolon” as classically defined by Srere (Srere and Knull, 1998; Watford, 1989). Studies by Solomonson *et al.* have demonstrated that exogenous citrulline was effective in stimulating nitric oxide production, suggesting that endothelial cells are able to



efficiently regenerate arginine from citrulline for nitric oxide production using the enzymes ASS and ASL (Ananthkrishnan, Barr, Summar, Smith, Kaplowitz, Cunningham, Magarik, Zhang, and Fike, 2009;Solomonson, Flam, Pendleton, Goodwin, and Eichler, 2003). These experiments support the hypothesis of a metabolon that includes the ASS, ASL and NOS enzymes with the substrate citrulline which is converted to arginine through the sequential actions of ASS and ASL and arginine is utilized as the substrate for nitric oxide production (Figure 2.3). Studies have shown that high basal levels of arginine do not increase nitric oxide production; a phenomenon known as the arginine paradox (McDonald, Zharikov, Block, and Kilberg, 1997;Tentolouris, Tousoulis, and Stefanadis, 2004), suggesting that citrulline may be more an effective substrate in eliciting an increase in nitric oxide production (Smith, Canter, Christian, Drinkwater, Scholl, Christman, Rice, Barr, and Summar, 2006) . Furthermore, Flam et al. have shown that nitric oxide synthase, ASS and ASL cofractionate with the caveolae, a subcompartment of the plasma membrane (Flam, Hartmann, Harrell-Booth, Solomonson, and Eichler, 2001). These results suggest a compartmentalized system for the production of nitric oxide from citrulline and provide evidence that the enzymes involved in nitric oxide production may interact at the molecular level in order to sufficiently channel substrate and produce nitric oxide (Figure 2.3). In our model, citrulline is circulated to tissues in the plasma. It is taken up by the neutral amino acid transporter, SN1, and is available for NO production through the sequential actions of ASS, ASL and a nitric oxide synthase. This model would explain the observation of sufficient arginine “pools” in the cell yet apparent substrate limitation for NO production, the

so call “arginine paradox” (McDonald *et al.*, 1997; Scalera *et al.*, 2009; Tentolouris *et al.*, 2004). Additionally, this model would predict that citrulline is an effective precursor for NO production.

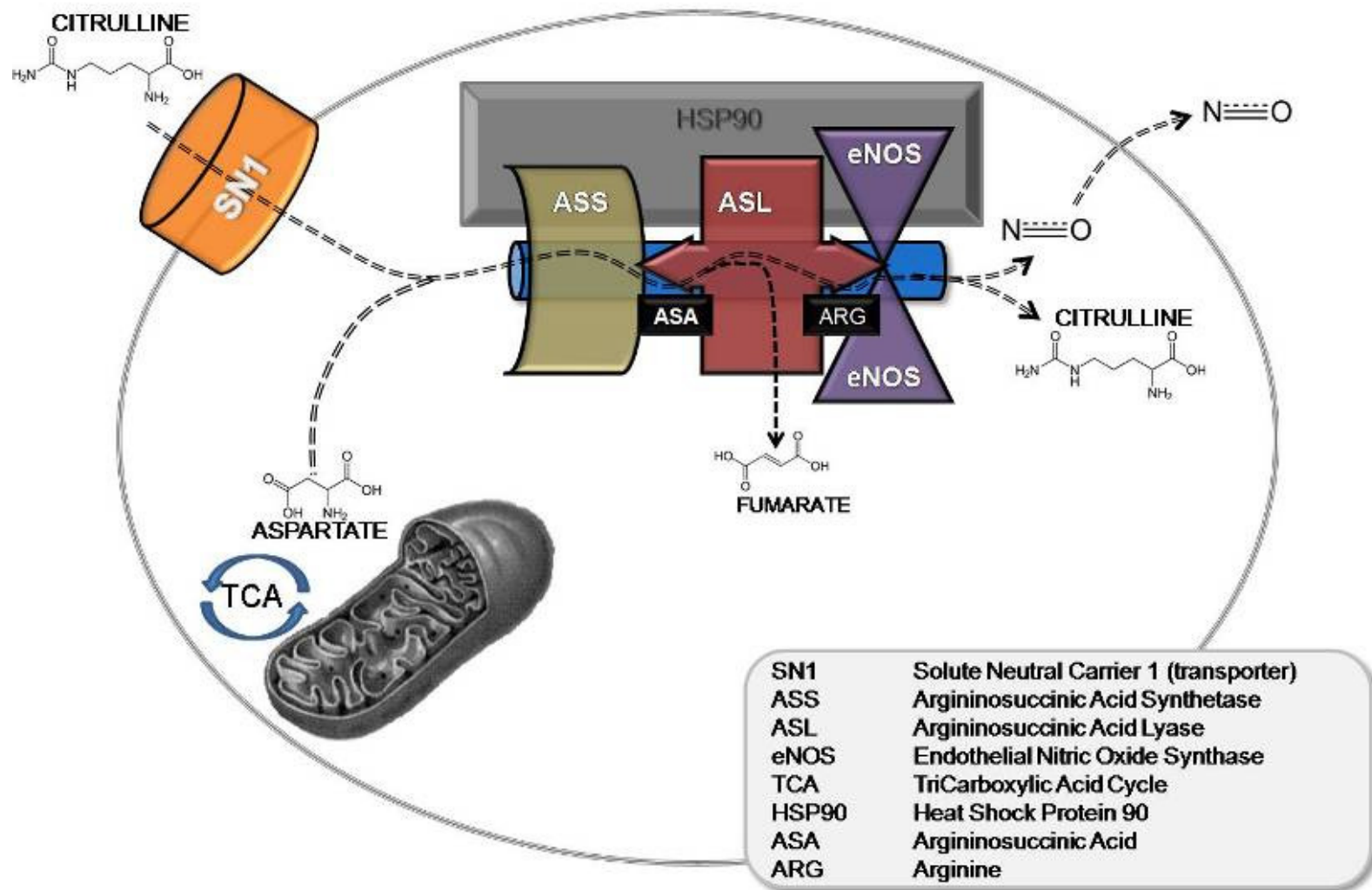


Figure 2.4 Model for Channeling and Enzyme Interactions in the Urea and Nitric Oxide Cycles

## **Pathophysiological Aspects**

### *Nitrogen Clearance Related Pathologies*

#### *Urea Cycle Disorders*

Urea cycle disorders include deficiencies of urea cycle enzymes. For example, Type 1 citrullinemia is a disorder caused by mutations in the *ASS* gene leading to reduced or abolished activity of the ASS enzyme. The disease is characterized by the accumulation of ammonia and citrulline and in the bloodstream. (Gao, Kobayashi, Tabata, Tsuge, Iijima, Yasuda, Kalkanoglu, Dursun, Tokatli, Coskun, Trefz, Skladal, Mandel, Seidel, Kodama, Shirane, Ichida, Makino, Yoshino, Kang, Mizuguchi, Barshop, Fuchinoue, Seneca, Zeesman, Knerr, Rodes, Wasant, Yoshida, De, Abdul, Begum, Horiuchi, Katunuma, Nakagawa, and Saheki, 2003). A large number of mutations have been identified in the *ASS* gene, many of which are proposed to disrupt protein structure (Engel, Hohne, and Haberle, 2009;Gao *et al.*, 2003;Karlberg, Collins, van den Berg, Flores, Hammarstrom, Hogbom, Holmberg, and Uppenberg, 2008;Mitchell, Ellingson, Coyne, Hall, Neill, Christian, Higham, Dobrowolski, Tuchman, and Summar, 2009). Although urea cycle disorders are rare, affecting 1 in 25,000 individuals, they result in high morbidity and mortality (Mitchell *et al.*, 2009;Summar, Dobbelaere, Brusilow, and Lee, 2008). Elevation of blood ammonia results in the accumulation of glutamine, causing encephalopathy and cerebral edema (Felipo and Butterworth, 2002;Summar *et al.*, 2008). Neurological dysfunction includes a range of neuropsychiatric and neurological symptoms such as altered mental status, abnormal motor function, impaired memory, seizures and coma (Ibarra-Gonzalez, Fernandez-Lainez, and Vela-Amieva,

2009;Summar *et al.*, 2008) . The clinical presentation and course of a urea cycle disorder is determined by the amount of urea cycle dysfunction. Partial enzyme function in combination with environmental factors can alter the age of onset and the course of the disease (Smith, Kishnani, Lee, Singh, Rhead, Sniderman, Smith, and Summar, 2005;Summar, Barr, Dawling, Smith, Lee, Singh, Rhead, Sniderman, and Christman, 2005). Complete absence of enzymatic activity and urea cycle function most often presents in the neonatal period and results in the most severe form of the disease. Patients with partial enzyme deficiency usually present with a hyperammonemic episode following an illness or stress. The treatment for urea cycle disorders includes reducing the plasma ammonia concentration, reducing the amount of nitrogen in the diet and preventing the body from producing excess nitrogen (Summar, 2001);(Gropman, Summar, and Leonard, 2007).

### *Nitric Oxide Related Pathophysiology*

#### *Immune Response*

Nitric oxide is produced by many cells of the immune system, including dendritic cells, natural killer cells, mast cells, neutrophils and macrophages in response to activation by cytokines and microbial compounds (Bogdan, 2001;Nathan, 1992). As part of the immune response, NO functions as a tumoricidal and antimicrobial molecule (Nathan, 1992). NO is also involved in T-helper cell deviation, the modulation and production of cytokines, chemokines and growth factors (Bogdan, 2001;MacMicking, Xie, and Nathan, 1997;MacMicking, Nathan, Hom, Chartrain, Fletcher, Trumbauer, Stevens, Xie, Sokol, Hutchinson, and .,

1995) . Given the diverse roles of NO in immune system function, dysfunction of NO has been associated with a variety of immune disorders. It is possible that the high amounts of NO released by immune cells to combat microbes can also damage healthy tissue. Studies have revealed an increase in nitric oxide in the plasma (Ueki, Miyake, Tominaga, and Eguchi, 1996), synovial fluid and cartilage of patients with rheumatoid arthritis (Grabowski, Wright, Van 't Hof, Helfrich, Ohshima, and Ralston, 1997). Today, both the protective and toxic effects of NO are still being investigated to develop therapeutic applications for diseases such as arthritis, colitis and inflammatory bowel disease (Bogdan, 2001;Jang and Murrell, 1998;Lundberg, Ehren, Jansson, Adolfsson, Lundberg, Weitzberg, Alving, and Wiklund, 1996;Lundberg, Hellstrom, Lundberg, and Alving, 1994;Lundberg, Lundberg, Alving, and Weitzberg, 1997). It is important for studies to consider the indelible link between the overlapping enzymes of the urea and nitric oxide cycles, ASS and ASL in the production of nitric oxide and its pathogenic and/or protective effects in autoimmune diseases.

### *Vascular Disease*

Nitric oxide plays a protective role in the vascular endothelium against atherosclerosis and thrombosis through the vasodilation of blood vessels and inhibition of platelet aggregation (Forstermann and Munzel, 2006). Several cardiovascular risk factors such as hypercholesterolemia, hypertension and smoking disrupt the function of the citrulline-NO cycle, leading to vascular diseases such as atherosclerosis (Goodwin, Pendleton, Levy, Solomonson, and Eichler,

2007;Harrison, 1997;Kinlay, Libby, and Ganz, 2001;Luscher and Barton, 1997;Vallance and Chan, 2001). Although the production of NO is directly related to the enzyme eNOS, overall production of eNOS by endothelial cells has been shown to be dependent on a functional citrulline-NO cycle (Goodwin, Solomonson, and Eichler, 2004;Xie and Gross, 1997). Hypertension is a multi-factorial disease where the exact pathogenic mechanisms are still being studied. Studies have shown that citrulline administration increases nitric oxide and ameliorates the development of hypertension in both newborn piglets (Ananthkrishnan *et al.*, 2009) and infants born with persistent pulmonary hypertension (Pearson *et al.*, 2001). These results suggest that the enzymatic function of the urea cycle is important in the production of nitric oxide in vascular disease. Studies have shown that intermediates from the urea/NO cycle in combination with genetic changes in the urea cycle affect responses to vasodilators such as bradykinin which employ NO mechanisms (Summar, Hall, Christman, Barr, Smith, Kallianpur, Brown, Yadav, Willis, Eeds, Cermak, Summar, Wilson, Arvin, Putnam, Wills, and Cunningham, 2004)

### *Neurological Disease*

Nitric oxide plays a role in several normal processes in the brain such as neurotransmission and synaptic plasticity, but has also been implicated in negative processes such as neurodegeneration and neuroinflammation (Dawson, Zhang, Dawson, and Snyder, 1994). Although the etiology and pathogenesis of neurodegenerative and neuroinflammatory disorders, such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis remain largely unknown, scientific

studies suggest that nitric oxide plays an important role through the generation of reactive oxygen species and oxidative damage (Bagasra, Michaels, Zheng, Bobroski, Spitsin, Fu, Tawadros, and Koprowski, 1995; Calabrese, Bates, and Stella, 2000; Chabrier, Demerle-Pallardy, and Auguet, 1999; Duncan and Heales, 2005; Knight, 1997). The nitric oxide pathway may play an important role in the sustained generation of endogenous nitric oxide that contributes to neurological disease. NO can be neurotoxic when triggered to be produced in excess by amyloid and elevated intracellular  $\text{Ca}^{2+}$  (Dawson *et al.*, 1994; Dawson and Dawson, 1996). Excessive NOS activity leads to increased oxidative stress, which in turn compromises cellular integrity and viability thereby contributing to neurodegenerative diseases through DNA damage, lipid peroxidation and energy depletion (Bolanos, Almeida, Stewart, Peuchen, Land, Clark, and Heales, 1997; Law, Gauthier, and Quirion, 2001). In Alzheimer's disease, studies have shown an increase in the mRNA transcripts for ASS and iNOS within the cortices of Alzheimer's disease patients (Haas, Storch-Hagenlocher, Biessmann, and Wildemann, 2002). Other studies have shown that both neurons and glial cells express ASS and ASL in parallel with iNOS, thereby giving these cells the enzymes necessary to produce endogenous nitric oxide and regenerate arginine for sustained nitric oxide production (Heneka, Wiesinger, Dumitrescu-Ozimek, Riederer, Feinstein, and Klockgether, 2001). The correlations between expression of ASS, ASL and NOS synthases in cells of patients with neurological disease is another result which suggests that the urea and nitric oxide cycle are linked at both the clinical and molecular levels.



**Significance:**

Over the last 20 years, a considerable body of evidence supports the idea that sequential enzymes within a metabolic pathway interact with each other to form highly organized complexes (Srere, 1987). These complexes contribute to an organism's ability to adapt to environmental changes at the molecular level.

Elucidating these interacting proteins is important for understanding the molecular regulation of disease pathologies associated with urea and nitric oxide production.

The present work represents a dedicated attempt to further characterize the enzymes involved in the urea and nitric oxide cycles, focusing on the overlapping cytoplasmic enzymes, ASS and ASL. The sequential actions of ASS and ASL are required to convert citrulline to arginine, which are both important substrates in the urea and nitric oxide cycles. The ASS and ASL enzymes are both localized to the cytoplasm where their sequential action is required in two metabolic pathways to produce important functional products. This work proposes that these enzymes interact as part of a metabolon to achieve their enzymatic goals. Because the urea and nitric oxide cycles are essential for homeostasis and functionality, deficiencies or excess of these important enzymes contribute to clinically significant diseases in many body systems. Similarly, variation in these enzymes leading to altered protein-protein interactions may contribute to urea cycle and nitric oxide cycle diseases/disorders. These studies will improve knowledge of enzyme expression and regulation in the molecular etiology of urea cycle and nitric oxide cycle diseases/disorders.

## **Hypothesis and Specific Aims**

### *Hypothesis*

The ASS and ASL enzymes are regulated in a coordinated and tissue-specific manner. These enzymes interact in multi-protein complexes whose components differ depending on cell type.

### *Specific Aim 1*

To characterize the context of ASS and ASL mRNA transcript expression, in relation to the other enzymes of the nitric oxide and urea cycles, in a panel of human tissues.

### *Specific Aim 2*

To determine the effects of known regulators of the urea and nitric oxide cycles on the *in vitro* expression of ASS and ASL mRNA transcript expression, protein levels and levels of the urea and nitric oxide cycle intermediates.

### *Specific Aim 3*

To demonstrate that ASS interacts with other proteins to form a multi-protein complex (metabolon).

## CHAPTER III

### CITRULLINE IS CORRELATED WITH INCREASED NITRIC OXIDE METABOLITES IN MURINE ANIMAL MODELS

#### Introduction

The urea cycle consists of a series of biochemical reactions that produce urea from ammonia. The urea cycle is the only metabolic pathway capable of converting nitrogen derived from protein intake and/or protein degradation into urea, which is then filtered by the kidney and excreted by the body. The complete urea cycle requires the activity of six enzymes: carbamoyl phosphate synthetase (CPSI), n-acetylglutamate synthetase (NAGs), ornithine transcarbamoylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL), arginase I (ARG I) and two transporters, citrin and ORNT1. The urea cycle is highly conserved in all mammalian species. Although several tissues express several urea cycle enzymes, only the liver has the full metabolic capacity of detoxifying ammonia to urea (Neill, Aschner, Barr, and Summar, 2009). The biochemical reactions of the urea cycle also produce two physiologically important metabolites, citrulline and arginine. These metabolites are used to build proteins and serve as substrates for nitric oxide production.

Nitric oxide (NO) is an important signaling molecule, modulating many aspects of vascular function such as the relaxation of smooth muscles, leukocyte activation, platelet aggregation and neurotransmission (Dawson *et al.*, 1994; Duncan *et al.*, 2005). NO is produced by endothelial cells and nonvascular cells. The nitric

oxide cycle (Figure 2.4) is composed of ASS, ASL, and one of the three nitric oxide synthases (endothelial (eNOS), inducible (iNOS), and neuronal (nNOS)) and uses citrin to provide the aspartate to condense with citrulline. Nitric oxide production is tightly regulated at both the transcriptional and post-transcriptional levels and also by substrate availability (Dawson *et al.*, 1994;Kone, 2001;Mori and Gotoh, 2000;Mount and Power, 2006;Sullivan and Pollock, 2003).

One approach to increasing NO synthesis is to provide additional substrate to the cells. L-arginine is the direct substrate for nitric oxide synthesis, but interestingly, studies have revealed that a lack of NO production occurs despite a high intracellular level of circulating arginine (Wu and Morris, Jr., 1998). The inability of cells to utilize the intracellular pool of arginine is termed the “arginine paradox” and research focused on determining the exact causes of this paradox are ongoing. The hypothesized mechanisms are: arginine analogs prevent arginine from being utilized by NOS through competition, or that arginine is compartmentalized which prevents NOS from accessing it (Scalera *et al.*, 2009;Tentolouris *et al.*, 2004). Citrulline can be utilized to generate and/or recycle arginine through the sequential actions of ASS and ASL. Studies have shown that intravenously administered citrulline ameliorates hypertension by increasing nitric oxide production (Ananthakrishnan *et al.*, 2009;Barr, Beverley, VanHook, Cermak, Christian, Drinkwater, Dyer, Raggio, Moore, Christman, and Summar, 2003;Barr, Tirona, Taylor, Rice, Arnold, Cunningham, Smith, Campbell, Canter, Christian, Drinkwater, Scholl, Kavanaugh-McHugh, and Summar, 2007;Pearson *et al.*, 2001;Summar, 2001;Summar *et al.*, 2004;Summar *et al.*, 2004). This study aims to characterize the molecular

relationship between mRNA expression, protein levels and the level of citrulline, arginine and nitric oxide in a wild-type mouse model. Use of this model organism was required to obtain tissue samples for RNA, protein, amino acid and nitric oxide analysis. ASS and ASL are hypothesized to be expressed in response to tissue needs for either nitrogen clearance or nitric oxide production, and that the concentrations of the intermediates/precursors of nitric oxide production (arginine and citrulline) and the secondary markers of nitric oxide (nitrates and nitrites) will reflect these tissue's roles and proposed metabolons.

## **Materials & Methods**

### *RNA Isolation*

Mouse tissues were harvested from B6 wild-type mice (n=3) and flash frozen in liquid nitrogen. mRNA was isolated using the RNA Midi Kit (Qiagen).

### *cDNA Preparation*

cDNA was prepared using the High Capacity Reverse Transcription Kit (Applied Biosystems). cDNA was synthesized in a 20- $\mu$ l mixture containing 1  $\mu$ g of DNase (Invitrogen) treated total RNA. Reverse transcription conditions were performed as suggested by the manufacturer with 10 min at 25 °C, 120 min at 37 °C and 5 sec at 85 °C. A sample lacking reverse transcriptase was used as a negative control.

### *Primers and Probes*

qRT-PCR was performed using the following Taqman Gene Expression Assays ordered from Applied Biosystems: ASS (Mm00711256\_m1), ASL (Mm01197741\_m1), GAPDH (4352932E), $\beta$ -actin (ACTB) (4352933E).

### *Relative Expression of mRNA Transcripts by qRT-PCR*

qRT-PCR analyses for ASS, ASL, GAPDH and B-actin mRNA were performed using the ABI PRISM 7700 Sequence Detection System and its analysis software, SDS 2.3 and RQ Manager (PE Applied Biosystems, Inc., Foster City, CA). PCR was performed with TaqMan Universal PCR Master Mix (Applied Biosystems) using 3  $\mu$ L of diluted (1:3) cDNA, 200 nmol/L of probe, and 300 nmol/L of primers in a 10- $\mu$ L final reaction mixture. Each of the 50 PCR cycles consisted of 15 s of denaturation at 95 °C and hybridization of probe and primers for 1 min at 60 °C. Each reaction was carried out in triplicate in at least 3 separate reverse transcriptase reactions. For each tissue, the relative expression of each gene was computed ( $34 - Ct^{\text{gene}}$ ), conservatively assuming that a Ct count of 34 reflects zero mRNA expression. Data are expressed as mean values  $\pm$  standard deviation (SD).

### *Western Blotting*

Protein was isolated from mouse tissue using Mammalian Protease Extraction Reagent (Pierce) supplemented with PMSF (Sigma) and Protease Inhibitor Cocktail (Sigma). Samples were homogenized using sonication. Protein concentration was determined using the BCA Protein Assay Kit (Pierce). 20  $\mu$ g of protein was loaded onto a 4-20% Tris-HCl Ready Gel (Bio-Rad) and separated by electrophoresis at

room temperature at 200 volts for 45 minutes. Protein was transferred to 0.45 micron nitrocellulose membranes (Bio-Rad) for 1 hour at 100 volts at 4°C. Membranes were blocked in 5% milk in TBS-T for 1 hour at room temperature and then probed with 1:1000 of appropriate primary antibody diluted in 5% milk in TBS-T overnight at 4°C. The membranes were washed 3 times with 0.5% TBS-T for 10 minutes and then incubated in 1:5000 dilution of secondary antibody diluted in 5% milk in TBS-T for 2 hours at room temperature. The membranes were washed 3 times with 0.5% TBS-T for 10 minutes. Proteins were visualized using ECL Western Blotting Substrate (Pierce). Each membrane was exposed to Kodak Bio-Max film (PerkinElmer) and developed. Scanning densitometry was calculated using TINA software (Raytest, Straubenhardt, Germany).

#### *Antibodies*

Primary antibodies for ASS, ASL and GAPDH were obtained from Santa Cruz Biotechnology. Anti-mouse and anti-goat were obtained from Promega.

#### *Amino Acid Analysis*

Analysis of amino acids was performed on protein-free extracts of mouse tissues. Briefly, tissues were flash frozen in liquid nitrogen and ground with a mortar and pestle in the presence of a 100  $\mu$ L of water: chloroform: methanol (3:5:12 v/v). Samples were transferred to a 1.5 mL centrifuge tube and centrifuged at 15,000 RPM for 15 minutes at 4°C. 3 successive extractions were performed, the supernants collected and pooled and frozen at -80°C. Amino acids were separated by cation-

exchange chromatography using a Hitachi L8800 amino acid analyzer (Hitachi USA, San Jose, CA). Levels of all detectable amino acids were measured after calibration of the analyzer. Data are expressed as  $\mu\text{Moles of Amino Acid} / \text{Total Protein } (\mu\text{g}/\mu\text{L})$  for each mouse studied.

#### *Nitric Oxide Analysis*

Analysis of nitric oxide production via the measurement of nitric oxide metabolites, nitrites and nitrates (NO<sub>x</sub>) was performed on a Sievers 280i Nitric Oxide analyzer. Nitric oxide is measured by nitrites and nitrates. Data are expressed as  $\mu\text{Moles NO}_x / \text{Total Protein } (\mu\text{g protein}/\mu\text{L})$  for each mouse studied.

#### *Statistical Analysis*

Measures of correlation were calculated using Pearson's correlation coefficient in Microsoft Excel with the assumption of no relationship,  $H_0 = 0$ .

## **Results and Discussion**

#### *mRNA Expression*

The ASS and ASL mRNAs were expressed in heart, liver, lung, kidney and spleen (Figure 3.1). In the liver, ASS and ASL play an important role in the urea cycle to process nitrogen, however we would have expected ASS and ASL to also be highly expressed in the heart as nitric oxide production is an important modulator of vascular function. The kidney is involved in the endogenous creation of arginine which also relies on the ASS and ASL enzymes (RATNER and PETRACK, 1953).



Nitric oxide production in the kidney is important in vascular hemodynamics, including the maintenance of vascular tone of the afferent and efferent arterioles (Schnackenberg, Patel, Kirchner, and Granger, 1997). Similarly, in the spleen, expression of nitric oxide is thought to invoke vascular dilation to improve flow through the splenic arteries (Vaillier, Daculsi, and Gualdel, 1996). Expression of ASS and ASL in the spleen would permit arginine to be generated, thereby continuing nitric oxide production.

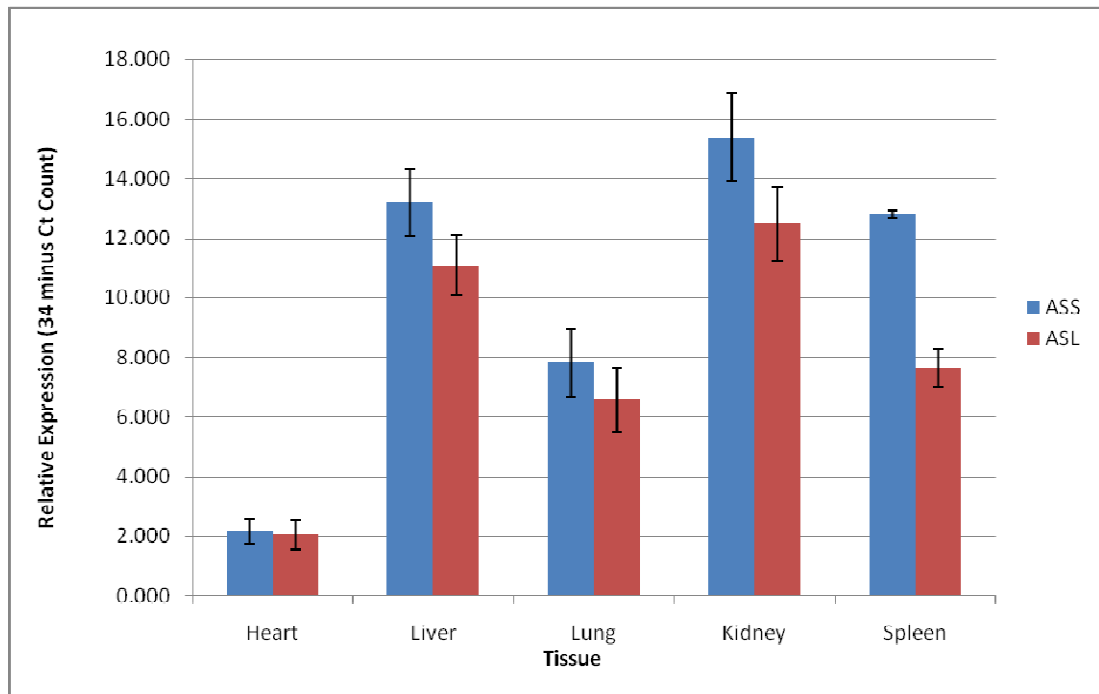


Figure 3.1 ASS and ASL mRNA Expression in Wild-Type Mouse Tissue. qRT-PCR results for ASS and ASL are expressed as the average relative counts (34 – Ct count)  $\pm$  standard deviation. Greater mRNA expression is indicated by greater relative expression. Experiments were repeated in triplicate.

### *Protein Expression*

ASS and ASL mRNA expression in relation to protein expression was examined in wild-type mouse tissue. CPSI and eNOS were also examined. ASS and ASL mRNA was detected in all of the tissues studied, with the highest expression in the kidney (Figure 3.1). Interestingly high amounts of ASS only in the liver and did not detect ASS expression in the other tissues we examined (Figure 3.2). ASL protein was most highly expressed in liver with small amounts detected in the spleen and heart. ASL was also detected in the lung and kidney tissues. Protein expression for ASL is very similar to its mRNA expression. It is interesting to note that both the mRNA and protein amounts for ASL are unexpectedly low for the heart tissue. It may be that the muscle tissue of the heart has little expression while it is higher in tissues surrounding the vascular structures. CPSI, a liver-specific protein, was utilized as a control. It is also interesting to note the amount of eNOS protein expression in all of the tissues studied. Named for its important role in endothelial cells, this data shows that eNOS protein is found in many tissues (Figure 4.2), which is supported by the literature (Feron, 1999; Searles, 2006).

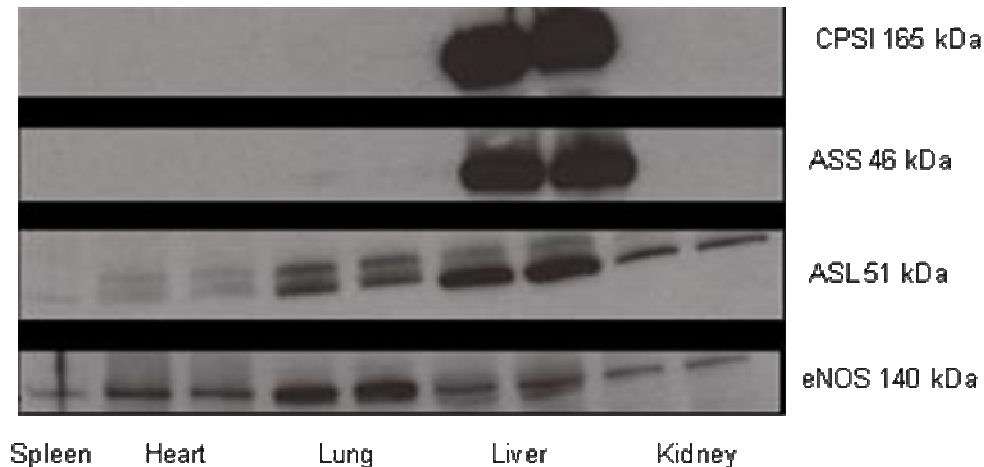


Figure 3.2 Expression of ASS, ASL, CPSI and eNOS Proteins in Wild-Type Mouse Tissue. 20  $\mu$ g of protein from respective tissues was used to examine expression of respective protein. Experiments were repeated in duplicate, except for the spleen which was only run in one experiment in Lane 1.

#### *Amino Acid Analysis*

We measured the concentrations of arginine and citrulline in mouse tissues (Figure 3.3). Theoretically tissues with more ASS and ASL enzymes should produce more citrulline, arginine and nitric oxide. Although ASS protein wasn't detected in all tissues, we expect that this is an artifact of these experiments as other studies have shown that ASS protein is expressed in the kidney thereby permitting the kidney to make arginine (Yu, Terada, Nagasaki, Takiguchi, and Mori, 1995). Arginine levels were highest in the kidney, which is an arginine generating organ and a therefore a good control for our experiments. Arginine was also high in the spleen and lungs, which had moderate levels of ASS and ASL mRNA expression and moderate levels of ASL protein expression. Interestingly, the concentration of citrulline is much lower in these tissues. The highest amount of citrulline was detected in the heart, which had the lowest expression of ASS and ASL mRNA as well as ASL protein.

This may be a result of complex regulation of the ASS and ASL enzymes or a mechanism involving citrulline transport into tissues.

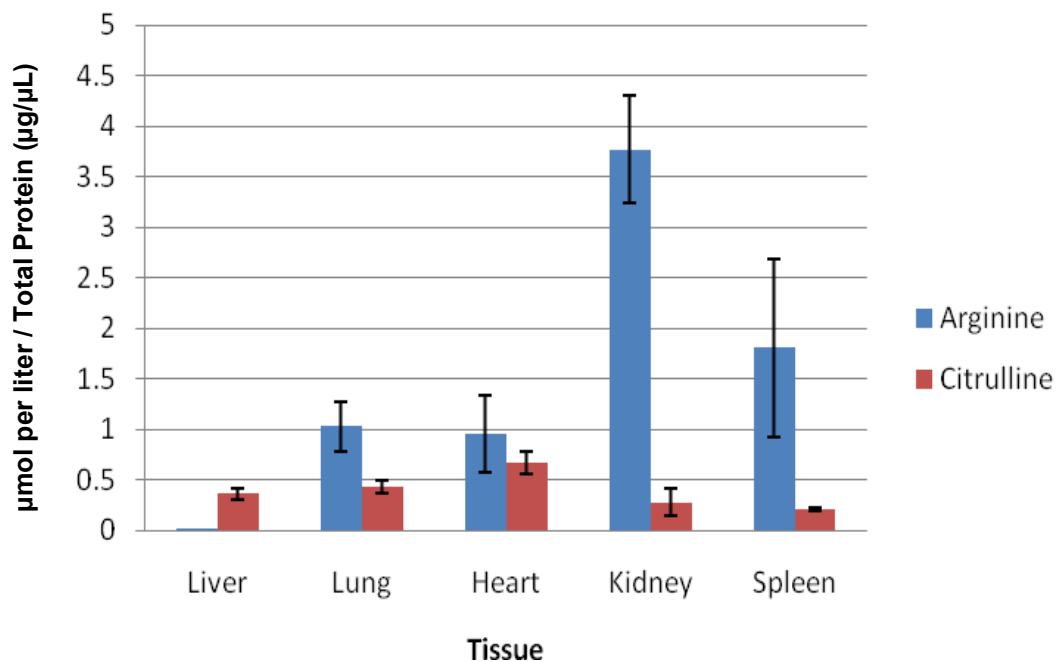


Figure 3.3 Arginine and Citrulline Concentrations in Mouse Tissues. Levels of urea cycle intermediates were measured using amino acid analysis and normalized to the concentration of protein from each tissue sample. Experiments were repeated in triplicate (n=3). Data are expressed as average  $\pm$  standard deviation.

### *Nitric Oxide Analysis*

Nitric has a very short half-life and is rapidly metabolized. Therefore, the analysis of nitric oxide typically consists of measuring the levels of nitric oxide metabolites or nitrites and nitrates. Nitric oxide metabolites were highest in the heart and lowest in the liver (Figure 3.4). The lung, kidney and spleen had moderate

concentrations of nitric oxide metabolites. It is interesting that the heart had the highest level of nitric oxide expression, despite low amounts of ASS and ASL mRNA and ASL protein. It is possible that the heart is able to utilize arginine directly for substrate but the heart had moderate levels of arginine. ASS and ASL may be regulated in a more complex manner than simply the expression at the mRNA and protein levels and the ability tissues to take up arginine and citrulline through transporters needs to be investigated in future experiments.

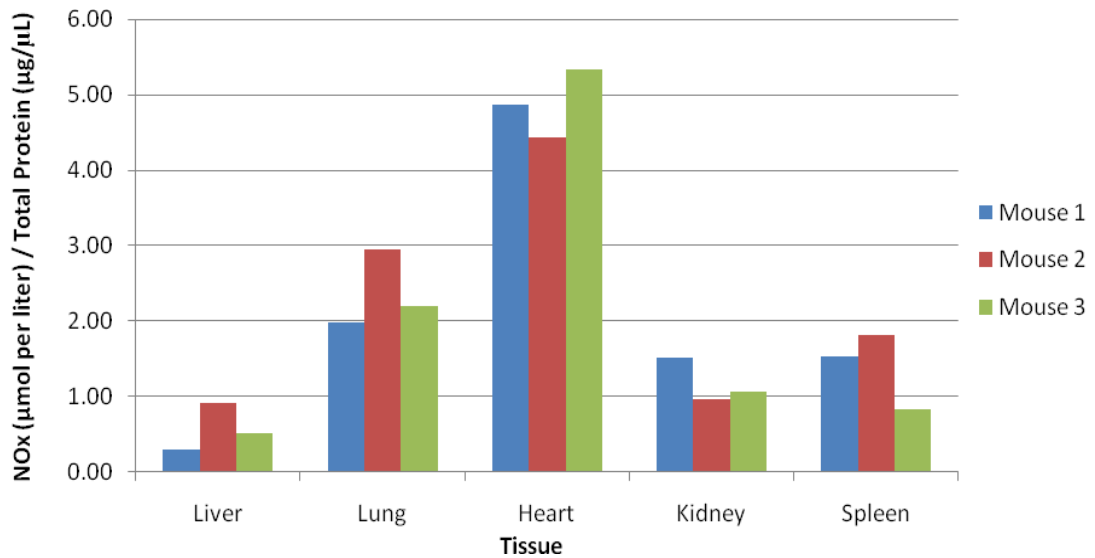


Figure 3.4 Analysis of Nitric Oxide Metabolites in Mouse Tissues, n=3. Nitric oxide was measured using nitric oxide analysis. Liver, lung, heart, kidney and spleen were studied in 3 different B6 mice.

### *Correlation of Citrulline and Arginine with Nitric Oxide Metabolites*

The hypothesis tested the relationship of citrulline and arginine with nitric oxide metabolites in mouse tissues. If arginine is the substrate utilized for nitric oxide production, the concentration of arginine in the tissue should correlate with nitric oxide metabolites present in that tissue. The concentrations of arginine and citrulline were graphed vs. the concentrations of nitric oxide (Figure 3.5 and Figure 3.6) and Pearson's correlation test was used to analyze the data. Arginine was not correlated with the concentration of nitric oxide metabolites in mouse tissues. The R-squared value was 0.0065 indicating no relationship between arginine concentration and nitric oxide metabolite concentration (Figure 3.5). Citrulline concentration was correlated with concentration of nitric oxide with an R-squared value of 0.6074 which had an associated p-value of 0.006 indicating that the relationship is significant (Figure 3.6). At the molecular level, this finding supports clinical studies which have shown that citrulline administration can increase nitric oxide production in patients with persistent pulmonary hypertension (Ananthakrishnan *et al.*, 2009; Smith *et al.*, 2006). At the molecular level, this may be a result of an increase in ASS and ASL expression or simply that citrulline may be the preferred substrate for nitric oxide production. The overarching hypothesis of this thesis is that nitric oxide production is regulated by an enzyme complex formed with ASS, ASL and NOS that citrulline may be the preferred substrate for entry into this hypothetical complex. Furthermore, we propose that the arginine generated by this complex would be utilized directly for nitric oxide production. These results may also play a role in understanding the "arginine paradox" where nitric oxide production can be

extremely low despite high intracellular levels of arginine. Further experiments elucidating the molecular roles of ASS and ASL in the production of nitric oxide need to be examined in tandem with analysis of the intermediates (arginine and citrulline) and end products (urea and nitric oxide).

**Summary:**

This study expands our understanding of the molecular basis behind the clinical finding that citrulline administration can ameliorate insufficient endogenous nitric oxide production. In this chapter we examined levels of mRNA and protein for ASS and ASL, the two enzymes that are involved in both the urea and nitric oxide cycles. Interestingly, this study revealed that in mouse tissues, citrulline concentration is associated with nitric oxide metabolite concentration. These studies were performed in a static model; it would be interesting to examine how the mRNA and protein levels change in response to stimuli. Additionally, measuring levels of intermediates and the final product (nitric oxide metabolites) provides an opportunity to examine the system as a whole to see if changes in mRNA or protein activity correlate with changes in intermediates and nitric oxide production.

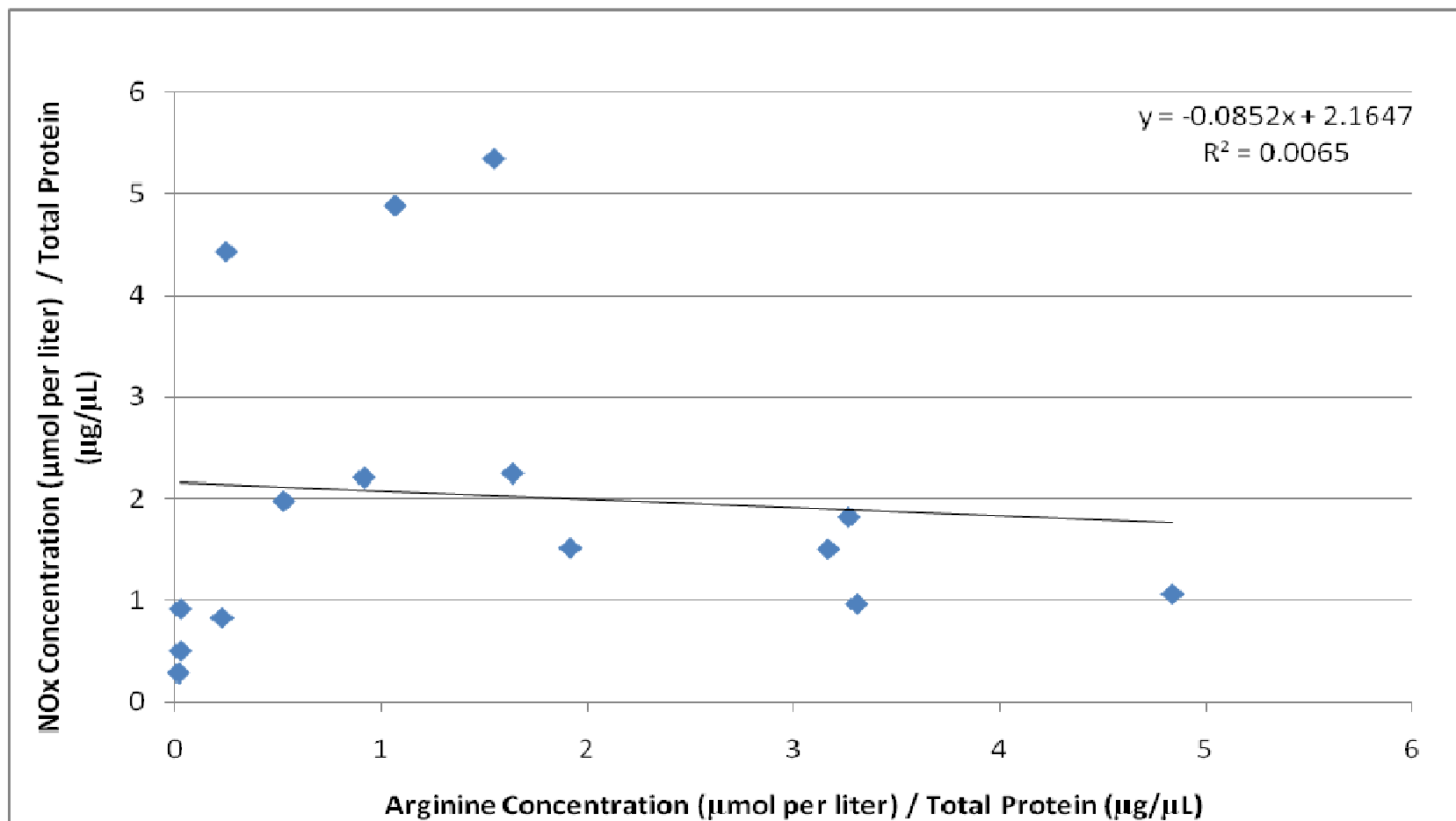


Figure 3.5 Relationship Between Tissue Arginine and Tissue Nitric Oxide Metabolites. For all values, the Pearson correlation coefficient was 0.0065. N=15.



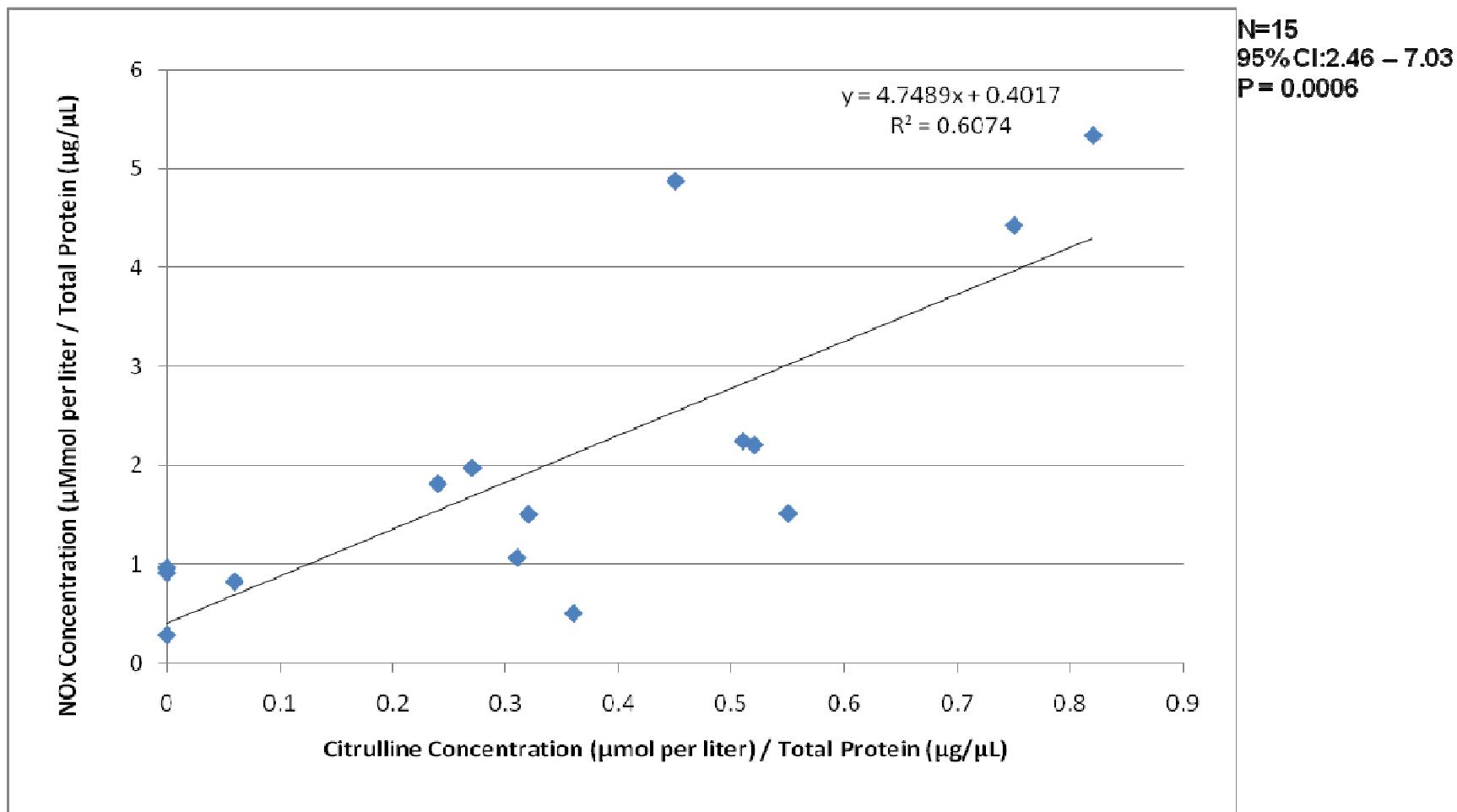


Figure 3.6 Relationship between Tissue Citrulline and Tissue Nitric Oxide Metabolites. For all values, the Pearson correlation coefficient was 0.6074 (p=0.0006). N=15.

## CHAPTER IV

### QUANTITATIVE RT-PCR COMPARISON OF THE UREA AND NITRIC OXIDE CYCLE GENE TRANSCRIPTS IN ADULT HUMAN TISSUES

This chapter is published under the same title in *Molecular Genetics and Metabolism*, June 2009. (Neill *et al.*, 2009)

#### Introduction

The urea cycle and nitric oxide cycle play significant roles in the complex processes of biochemical and physiologic reactions. These cycles have distinct biochemical goals: clearance of waste nitrogen, the production of the intermediates ornithine, citrulline, and arginine for the urea cycle, and the production of nitric oxide for the nitric oxide pathway. Despite their disparate functions, the two pathways share two enzyme components (ASS and ASL) and a transporter (citrin) encoded from single genes. The expression of the nitric oxide synthases in combination with enzymes of the urea cycle is critical in determining the global metabolic potential of the urea and nitric oxide cycles in different human tissues. This is especially important in diseases where urea cycle and nitric oxide cycle function may be impaired.

Moderate to severe genetic defects in any of the genes that code for urea cycle enzymes result in a urea cycle disorder (UCD) (Krebs and Henseleit, 1932). Previous studies from our laboratory have shown that, in addition to causing UCDS, a quantitative change in urea cycle enzyme expression and function can affect nitric oxide production by limiting substrate availability (Barr *et al.*, 2007;Canter,

Summar, Smith, Rice, Hall, Ritchie, Motsinger, Christian, Drinkwater, Jr., Scholl, Dyer, Kavanaugh-McHugh, and Barr, 2007). The broad and complex physiological functions of the enzymes shared by these two pathways present challenges in a variety of clinical conditions ranging from UCDs to post-cardiac surgery pulmonary hypertension, necrotizing enterocolitis, persistent pulmonary hypertension of the newborn, and bone marrow transplant related hepatic veno-occlusive disease (Barr *et al.*, 2003;Kallianpur, Hall, Yadav, Byrne, Speroff, Dittus, Haines, Christman, and Summar, 2005;Pearson *et al.*, 2001;Summar *et al.*, 2004;Summar *et al.*, 2004). Given the complex roles of the urea cycle and nitric oxide cycle enzymes, we hypothesized that we would be able to detect differences in gene expression in different tissues and that specific enzymes would be co-expressed together, reflecting the overall function of these enzymes in a specific tissue. To test our hypothesis, we investigated mRNA transcript expression using quantitative polymerase chain reaction (qRT-PCR) of the main enzymes of the urea and nitric oxide cycles, including CPSI, NAGs, OTC, ASS, ASL, ARG1, ARG2, CITRIN, ORNT1, eNOS, iNOS and nNOS, in a panel of normal human tissues. Our results showed that we were able to measure quantitatively, urea and nitric oxide cycle enzyme expression in each tissue. Furthermore, we were able to group co-expressed genes in order to determine a global view of the function of these enzymes in a particular tissue. This study provides an interesting view of tissue differences in the available transcripts for the different genes. In diseases where urea and/or nitric oxide metabolism is impaired, our results show that it is important to examine not

only the expression of each individual enzyme but the context of the co-expression of enzyme groupings in different tissues.

## **Materials and Methods**

### *RNA from Human Tissue*

Total human RNA from normal tissue samples from small intestine, ileum, liver, pancreas, kidney, brain, testis, skeletal muscle, spleen, heart, and lung were obtained from Stratagene and Chemicon. The RNA was isolated from two healthy deceased donors using a modified guanidinium thiocyanate method ([www.stratagene.com](http://www.stratagene.com)). RNA was tested by spectroscopy to verify concentration and purity.

### *cDNA Preparation*

cDNA was prepared using the High Capacity Reverse Transcription Kit (Applied Biosystems). cDNA was synthesized in a 20- $\mu$ l mixture containing 1  $\mu$ g of DNase (Invitrogen) treated total RNA from each tissue sample. Reverse transcription conditions were performed as suggested by the manufacturer for # cycles of 10 min at 25 °C, 120 min at 37 °C and 5 s at 85 °C. A negative control consisted of a sample lacking reverse transcriptase.

### *Primers and Probes*

qRT-PCR was performed using the following Taqman Gene Expression Assays ordered from Applied Biosystems: CPSI (Hs00919483\_m1), NAGs

(Hs00400246\_m1), OTC (Hs00166892\_m1), ASS (Hs00540723\_m1), ASL (Hs00163695\_m1), ARG1 (Hs00968979\_m1), ARG2 (Hs00265750\_m1), CITRIN (Hs00185185\_m1), ORNT1 (Hs01111100\_m1), eNOS (Hs00167166\_m1), iNOS (Hs00167257\_m1), nNOS (Hs00167223\_m1), GAPDH (4333764F),  $\beta$ -actin (ACTB) (4333762F).

#### *Relative Expression of mRNA Transcripts by qRT-PCR*

qRT-PCR analyses for CPSI, NAGs, OTC, ASS, ASL, ARG1, ARG2, CITRIN, ORNT1, eNOS, iNOS, nNOS, GAPDH and B-actin mRNAs were performed using the ABI PRISM 7700 Sequence Detection System and its analysis software, SDS 2.3 and RQ Manager (PE Applied Biosystems, Inc., Foster City, CA). PCR was performed with TaqMan Universal PCR Master Mix (Applied Biosystems) using 3  $\mu$ L of diluted (1:3) cDNA, 200 nmol/L of probe, and 300 nmol/L of primers in a 10- $\mu$ L final reaction mixture. Each of the 50 PCR cycles consisted of 15 s of denaturation at 95 °C and hybridization of probe and primers for 1 min at 60 °C. Each reaction was carried out in triplicate using the same RNA source in at least three separate reverse transcriptase reactions for quality control and redundancy (at least nine qRT-PCRs per gene).

#### *Data Analysis*

The cycle threshold (Ct) values were determined using the software package for qRT-PCR analysis provided by ABI (PE Applied Biosystems, Inc., Foster City, CA). The Ct value reflects the PCR cycle number at which the fluorescence

generated within the reaction exceeds the threshold (background fluorescence) and is inversely proportional to the amount of transcript present in a sample. Thus, the larger the Ct value the less detectable message is present to act as template. While a Ct value for no transcript cannot be determined in these samples, the value for extremely low or single copy presence is set at a Ct value of  $\geq 34$  (ABI publication cms-042380), which is based upon data from a large number of genes and reference curves. The quality and reproducibility of the reactions was determined by calculating the standard deviation of the Ct value on at least three reactions. A standard deviation value  $\leq 0.250$  indicates a 95% probability of detecting a two-fold dilution or increase in template (1 Ct). Since this experiment did not compare changes in RNA from a basal state in the tissues, a Delta Delta Ct was not calculated. The Ct values for the control transcripts (GAPDH and B-actin) are shown as a demonstration of integrity of the RNA. Since these control genes vary in expression between tissues, they were not used for standardization between RNA sources. Changes in cycle count do not reflect linear changes; therefore, we have tried to avoid further mathematical manipulation of the data. For graphical display and ease of understanding, we subtracted the Ct value from 34 (cutoff for no template) which presents the samples with the most starting template (lowest Ct) as the largest numbers. We do not compare Ct values between genes, since the relative efficiency of the qRT-PCR reactions for each gene makes such data difficult to interpret. For graphical display of the Ct values in Figure 3.3, the line thickness for each transcript is reflective of the (34-Ct) value for each transcript in each tissue.

## Results and Discussion

As a central hub of basic biochemistry and physiology, the expression of urea and nitric oxide cycle enzymes will affect many clinical disease states (Barr *et al.*, 2003; Kallianpur *et al.*, 2005; Pearson *et al.*, 2001; Summar *et al.*, 2004; Summar *et al.*, 2004; Wang, Newton, and Marsden, 1999). We were able to amplify mRNA transcripts coding for each gene in the urea and nitric oxide cycles, including the transporters ORNT1 and citrin, from eleven different human tissues. A graphical representation of the Ct values for each gene is presented in Figure 4.1. The Ct values and standard deviations for each gene were consistent across multiple reverse transcriptions and qRT-PCRs in (Table 4.1). To better visualize the patterns of co-expressed genes, we generated a graphic of the urea and nitric oxide cycles and assigned the  $(34 - Ct)$  value to the line thickness for each gene in each tissue Figure 4.2. Dotted lines indicate no significant transcript detection. The gene expression patterns show trends that are consistent with the traditional physiologic roles associated with these specific tissues (Figure 4.1 and Figure 4.2). The RNA samples studied are from whole organ preparations and as such, represent a global view of metabolic activity, and do not reflect gene expression at the cellular level. The expression patterns measured in this study are not necessarily reflective of protein levels in each tissue. Although the regulation of protein abundance is not completely determined by mRNA expression, differences in metabolic activity have been correlated with the mRNA levels of many genes. Therefore, these co-expressed genes may affect molecular protein production and physiologic functions. Examples of co-expression affecting physiologic expression are described below.

Raw Ct Values Obtained from qRT-PCR Assays														
Tissue	CPSI	NAGs	OTC	ASS	ASL	ARG1	CITRIN	ORNT1	ARG2	eNOS	iNOS	nNOS	GAPDH	B-actin
Liver	22.85±0.49	27.51±0.09	25.07±0.13	20.75±0.37	24.50±0.23	22.56±0.06	25.30±0.11	22.91±0.40	28.50±0.14	29.38±0.30	37.08±0.30	34.88±0.25	25.75±0.08	20.36±0.18
Small Intestine	25.86±0.30	26.43±0.17	25.42±0.30	23.91±0.53	25.53±0.27	39.53±0.41	27.23±0.40	29.95±0.47	24.32±0.48	32.27±0.44	30.64±0.36	32.36±0.25	22.88±0.35	19.11±0.47
Ileum	25.31±0.35	27.33±0.29	26.45±0.45	23.03±0.44	25.43±0.45	33.79±0.41	27.98±0.19	26.37±0.11	25.84±0.43	29.53±0.41	26.09±0.20	31.10±0.21	21.17±0.31	19.31±0.17
Pancreas	26.96±0.49	32.57±0.10	31.86±0.08	23.44±0.47	27.61±0.41	34.94±0.48	27.03±0.13	25.36±0.27	26.47±0.16	28.31±0.35	34.36±0.44	29.04±0.33	20.16±0.13	19.62±0.20
Kidney	32.31±0.16	28.73±0.45	36.29±0.06	21.53±0.55	27.08±0.25	36.42±0.49	27.38±0.47	27.64±0.12	23.88±0.33	30.61±0.44	37.55±0.47	29.96±0.47	23.99±0.52	20.15±0.48
Brain Stem	34.02±0.39	31.09±0.19	36.12±0.34	27.19±0.23	29.08±0.12	31.69±0.10	26.37±0.11	29.47±0.16	27.11±0.36	29.45±0.27	32.61±0.39	27.59±0.43	23.83±0.20	20.22±0.07
Testis	32.03±0.50	33.58±0.13	36.08±0.27	26.28±0.37	27.80±0.11	33.39±0.10	29.18±0.42	30.85±0.33	29.01±0.30	33.09±0.41	35.28±0.47	30.93±0.33	22.42±0.23	21.25±0.20
Skeletal Muscle	34.53±0.19	38.41±0.30	36.95±0.36	26.67±0.43	29.70±0.45	35.16±0.41	32.99±0.55	28.18±0.03	27.65±0.24	33.23±0.32	38.08±0.44	24.54±0.53	21.41±0.48	22.27±0.35
Spleen	33.17±0.18	34.74±0.43	36.21±0.08	28.49±0.27	26.41±0.46	31.44±0.45	27.78±0.43	28.18±0.03	30.18±0.14	28.09±0.16	38.35±0.44	36.16±0.38	23.35±0.02	18.62±0.12
Heart	30.99±0.48	31.20±0.48	33.30±0.21	22.54±0.26	26.36±0.40	27.97±0.03	27.24±0.40	28.51±0.28	25.56±0.14	28.01±0.32	37.39±0.36	34.27±0.33	17.81±0.06	19.70±0.24
Lung	30.66±0.14	31.76±0.48	30.98±0.51	24.36±0.12	26.92±0.25	27.56±0.08	27.97±0.08	29.74±0.27	29.24±0.23	28.21±0.44	32.13±0.53	29.11±0.25	20.41±0.21	22.93±0.31

Table 4.1 Raw Ct Values for Urea and Nitric Oxide Cycle Enzymes in Normal Human Tissue.



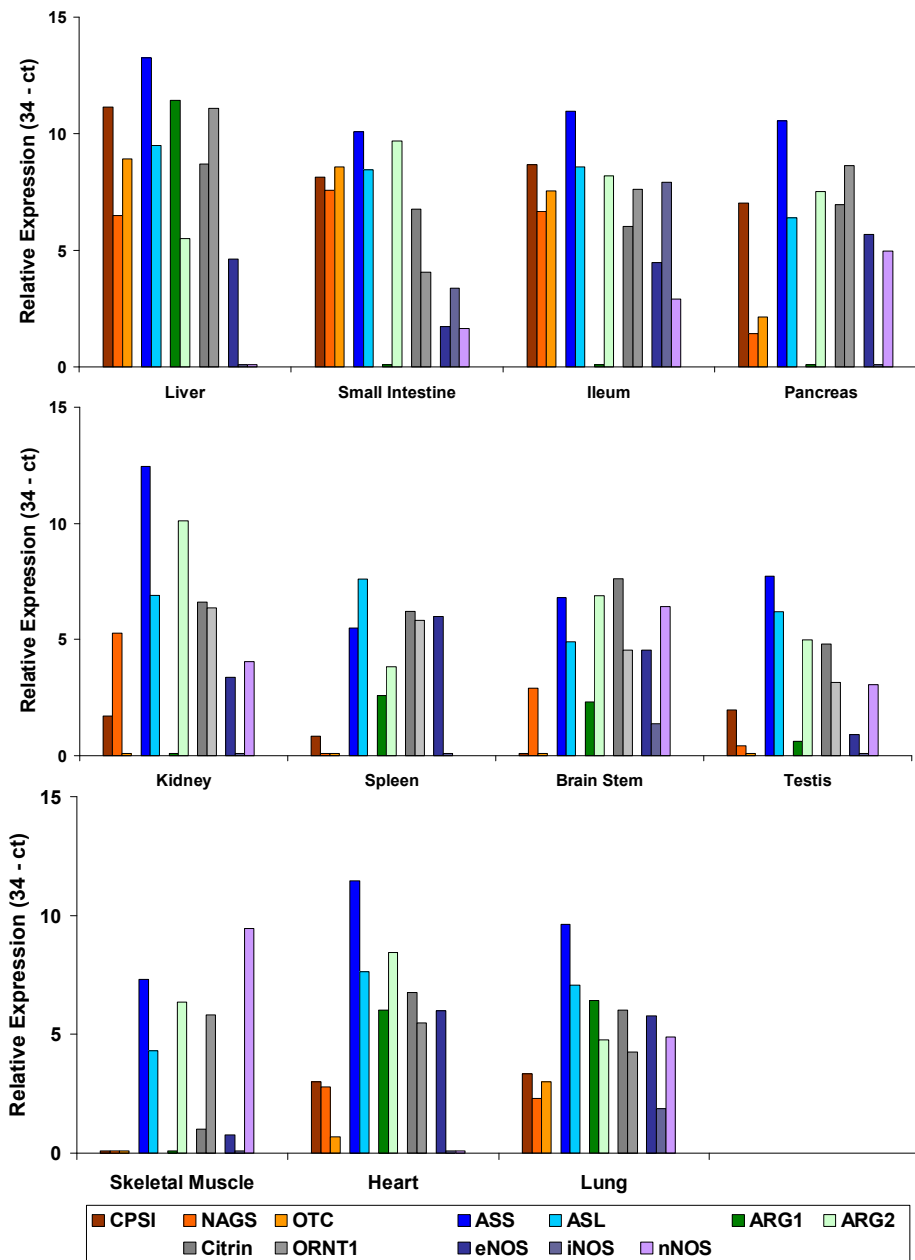


Figure 4.1 Ct values for transcripts expressed as (34-Ct) using 34 as a theoretical “no-expression” value for qRT-PCR. Genes are grouped by their position or function in the cycle. Tissues are roughly grouped by the functional patterns observed. All determinations were performed from at least three independently prepared RNA isolations.

### *Urea Production*

We detected the expression of the full complement of urea cycle enzymes only in the liver tissue. More specifically, we observed marked elevations of CPSI, NAGS, OTC, ASS, ASL, ARG1, CITRIN, and ORNT1 transcripts with minimal increase in the ARG2 transcript. The small intestine expressed all of the enzymes except for ARG1. This may explain why despite the majority of the urea cycle enzymes being present in the small intestine and ileum, these tissues are not able to compensate for the ammonia clearance capacity lost as a result of chronic liver disease, such as cirrhosis.

### *Arginine and Citrulline Production*

Arginine and citrulline production are crucial for the function of both the urea and nitric oxide cycle. Arginine serves as a substrate in both cycles. We observed elevations of CPSI, NAGS, OTC, ASS, ASL, and CITRIN transcripts with minimal increases in ARG1 and ORNT1 transcripts. Expression of this complement of enzymes would be sufficient to carry nitrogen through the upper and middle urea cycle to generate citrulline or arginine. These intermediates may be exported to other tissues for protein synthesis, nitric oxide production, or polyamine production. We observed this pattern in the small intestine and the ileum, which is consistent with the clinical need to provide supplemental arginine and citrulline in patients with urea cycle disorders even after liver transplantation (Tuchman, 1989; Tuchman, Mauer, Holzkecht, Summar, and Vnencak-Jones, 1992). Studies in animals have shown that the small intestine is a net exporter of citrulline. Citrulline can be converted to

arginine, either in the kidney or the small intestine itself, where we have observed sufficient amounts of ASS and ASL transcripts (Windmueller and Spaeth, 1981). Expression of this complement of enzymes would supply substrates for numerous biochemical reactions (NO production, polyamine production, protein production, etc.).

#### *Nitric Oxide Metabolism*

Nitric oxide has many physiologic functions involving the cardiovascular, immune, and neurological systems. The expression of eNOS, iNOS, or nNOS was detected in all tissues except testis and kidney. The liver, ileum, small intestine, and brain stem had elevated ASS and ASL transcripts coupled with co-expression of one of the NOS enzymes, thus providing these tissues with the capability to generate nitric oxide. The heart, lung, spleen, and pancreas had elevated ASS or ASL transcripts, but not both. It is known that the heart, lung and spleen are nitric oxide producers so our results imply that these tissues obtain arginine substrate by importing it or that the nitric oxide cycle in these tissues is regulated above the level ASS and ASL gene expression.

#### *Polyamine Production, Urea Production, or Arginine Competition*

Arginase enzymes are capable of producing ornithine for the production of polyamines. Therefore, we would expect that the co-expression of ORNT1 and either ARG1 or ARG2 without elevation of the transcripts from the first part of the cycle (CPSI, OTC, NAGS) would permit polyamine production. We detected high levels

of ARG2 and ASL transcripts in the kidney, moderate expression of ASS and ORNT1 transcripts and low levels of eNOS and nNOS transcripts. We suggest that the expression of this specific complement of enzymes in the kidney may be involved with functional osmolarity during filtration. It should be noted that we did not detect ARG1 transcript in the kidney in our study, which contradicts early reports of ARG1 presence in kidney (Yu, Yoo, Aguirre, Tsoa, Kern, Grody, Cederbaum, and Iyer, 2003). The lung, heart, and spleen had elevated expression of ARG1 but lacked expression of the enzymes from the upper portion of the urea cycle (CPSI, OTC, NAGS). The expression of ARG1 in these tissues may provide a balance for the elevated amounts of NOS transcripts seen in these tissues by scavenging arginine. This mechanism has been proposed as a regulation of NOS by a number of studies (Hallemeesch, Lamers, and Deutz, 2002;Kepka-Lenhart, Ash, and Morris, 2008;Meurs, Maarsingh, and Zaagsma, 2003;Mori *et al.*, 2000;Nelin, Wang, Zhao, Chicoine, Young, Hatch, English, and Liu, 2007;Ricciardolo, 2003). Further studies examining metabolite processing coupled with enzyme expression at the cellular level is required to understand better these co-expression patterns.

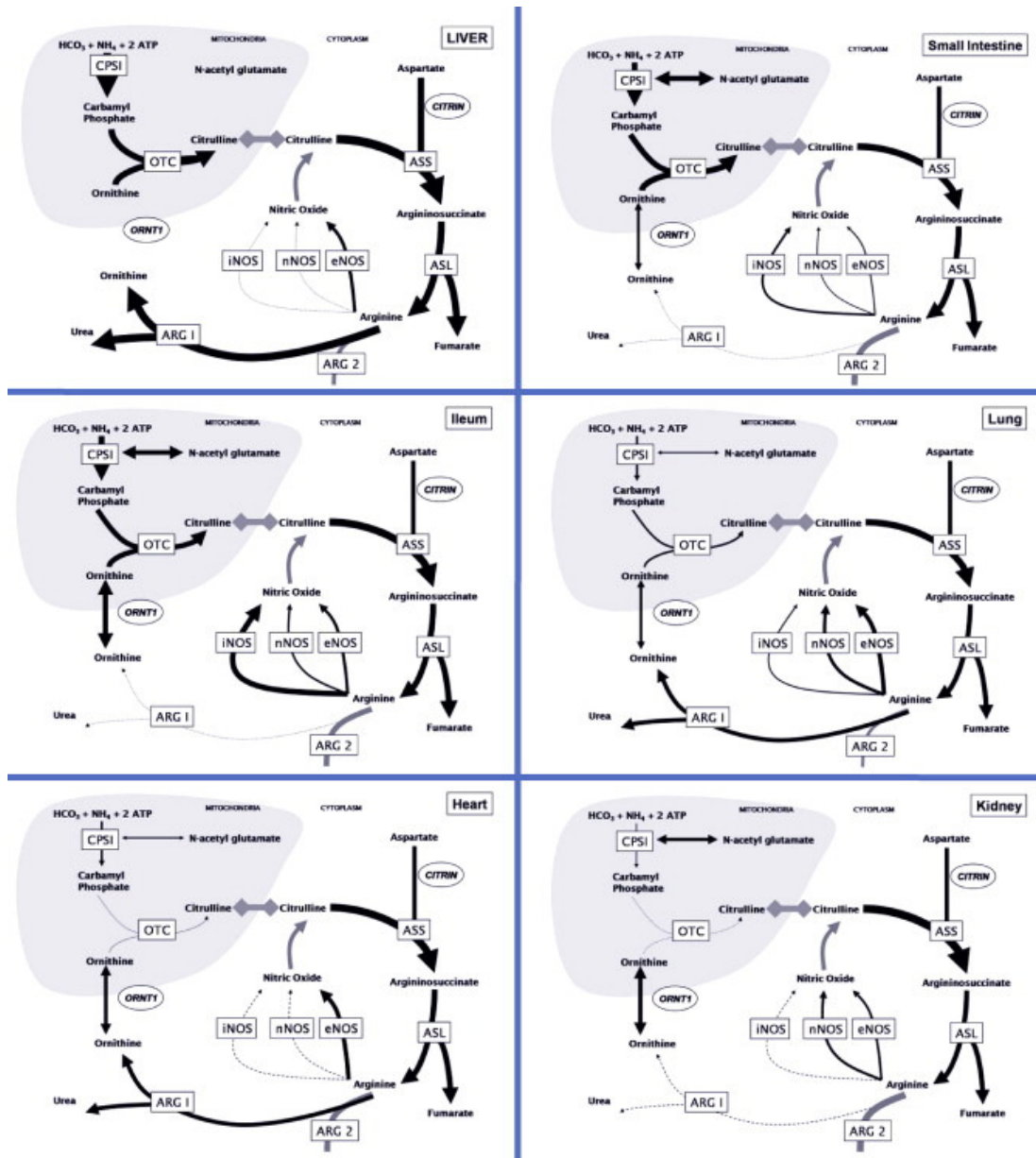


Figure 4.2 Pathway Representations of Relative Expression Levels by Tissue  
 The line thicknesses reflect the (34-Ct) expression level for each gene with thicker lines denoting greater RNA transcript levels. Dotted lines indicate that the (34-Ct) value was 0 or less than 0 thus indicating no significant amount of transcript.

## Summary

As hypothesized, we were able to distinguish co-expression patterns for the enzymes of urea and nitric oxide cycles in human tissues. Overall, these results suggest that there are at least three functional patterns for co-expressed enzymes including the production of urea, the production of citrulline/arginine and ornithine, and the production of nitric oxide for signaling uses. At a broader level, and with the discovery that the number of human genes is less than predicted by function, the co-expression of the enzymes in these pathways exemplifies an economical use of genetic code. Our co-expression data also suggests that these enzymes may share common regulation at the transcriptional level. While the double duty of many of the enzymes in this pathway is efficient, it also makes these pathways biologically vulnerable. Thus, ammonia clearance, arginine/citrulline production, nitric oxide metabolism, creatine production, polyamine production, and urea production are all vulnerable to genetic defects in this system. This may explain the high degree of evolutionary conservation seen in these genes, and the serious effects of genetic defects in any of these gene (Jackson, Beaudet, and O'Brien, 1986). For example, in patients with rare inborn errors of urea cycle function, we have observed complications such as hypertension which go beyond those explained by straightforward hyperammonemia and lack of arginine production (Butterworth, 1998; Caldovic, Morizono, Panglao, Lopez, Shi, Summar, and Tuchman, 2005; Gropman *et al.*, 2007; Morris, Morris, Jr., Hagar, Van Warmerdam, Claster, Kepka-Lenhart, Machado, Kuypers, and Vichinsky, 2003; Pita, Wakabayashi, Fernandez-Bustos, Virgili, Riudor, Soler, and Farriol, 2003; Summar, 2001; Summar

*et al.*, 2005). The intertwining nature of the urea and nitric oxide cycles in multiple tissues may be one of the reasons for this (Barr *et al.*, 2003; Hayashi, Juliet, Matsui-Hirai, Miyazaki, Fukatsu, Funami, Iguchi, and Ignarro, 2005; Pita *et al.*, 2003; Smith *et al.*, 2006; Summar *et al.*, 2004; Summar *et al.*, 2004). As these studies progress, transcriptional regulation, tissue specificity, and the localization of the full complement of enzymes must be considered in patients with classic urea cycle defects. Future studies will examine the change in the relationships of these genes under stress or stimulus conditions. The results of this study indicate the need for a change in the way we consider classic inborn errors of urea cycle metabolism and the degree of complexity they present.

## CHAPTER V

### EFFECTS OF EXTRACELLULAR STIMULI ON THE mRNA AND PROTEIN EXPRESSION OF ENZYMES IN THE UREA AND NITRIC OXIDE PATHWAYS AND THE LEVELS OF PATHWAY INTERMEDIATES

#### Introduction

A primary objective in the study of the urea and nitric oxide cycles is to determine where, when and how the individual enzymes are regulated. In this chapter, the effects of known regulators of the urea and nitric oxide cycles on ASS and ASL mRNA transcript expression, protein expression and the levels of pathway intermediates (arginine and citrulline) are examined. We hypothesized that ASS and ASL transcripts and protein would be expressed at similar levels and that ASS and ASL would be expressed in similar tissues.

Argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) are two enzymes that are essential in both urea and nitric oxide metabolism. Deficiencies of either of these enzymes results in a disordered urea cycle as well as disordered nitric oxide production. Little is known about the correlation between mRNA and protein in the urea and nitric oxides. In rats treated with dexamethasone hepatic urea cycle enzymes increased in mRNA abundance as measured by dot-blot and quantitative northern blot analyses (Morris, Jr., 1992). However, recent studies on urea cycle enzymes have shown that despite increases in their mRNA levels, there was no corresponding increase in overall urea cycle activity (Iwata, Kajimura, and Sakamoto, 2000;Kong, Edberg, Korte, Salo, Wright, and Anderson, 1998).



Greenbaum et al. suggest that at a general mechanistic level, those poor correlations of mRNA levels with protein levels are due to complicated post-transcriptional mechanisms and differential *in vivo* protein half-lives (Greenbaum, Colangelo, Williams, and Gerstein, 2003). The objective of this study was to undertake an in-depth analysis of the mRNA and protein expression of the overlapping urea cycle and nitric oxide cycle enzymes and intermediates in a cell culture model. The liver plays a central role in metabolism, including nitrogen metabolism. Human tissue-derived cell lines are frequently used as a tool for *in vitro* urea cycle metabolism studies. Hepatoma HepG2 cells are frequently used although studies have yet to determine levels of the urea cycle enzymes and intermediates in this cell line. Furthermore, data of urea cycle enzyme expression in other cell lines is limited. In the present study, the expression of human urea cycle enzymes in human is studied in tissue-derived cell lines. The goal of this Chapter was to determine the relationships between mRNA expression, protein expression and levels of intermediates at the molecular level to investigate how stimuli affect these relationships and ultimately, the production of nitric oxide.

## **Materials and Methods**

### *Cell Line Selection*

Based on the results from Chapter III, cell lines were chosen based on differential expression of the enzymes from tissues where ASS and ASL have different biological roles. HEPG2 liver hepatoma cells were chosen because this cell line expresses the full complement of urea cycle enzyme transcripts. HEK kidney

cells were chosen because this cell line lacks ARG1, which would allow the sequential actions of ASS and ASL to lead to arginine production. The kidney is a known producer of arginine. Finally, HUVEC primary umbilical endothelial cells were chosen because this cell line is known to produce nitric oxide.

#### *Cell Culture Conditions*

HEPG2 cells were grown in DMEM/F12 medium (Gibco) supplemented with 5% fetal bovine serum and 100 µg /mL of penicillin-streptomycin. HEK cells were grown in DMEM High Glucose Medium (Gibco) supplemented with 5% fetal bovine serum and 100 µg/mL of Anti-Penicillin Anti-Streptomycin (Gibco). HUVEC cells (Lonza) were grown in EGM Endothelial Growth Medium and supplemented with EGM SingleQuot Kit Supplements and Growth Factors (Clonetics). All cells were grown in an incubator at 37°C with 5% carbon dioxide (NuAIRE).

#### *Stimuli*

Cells were split and plated at 70% confluency in T-150 flasks (Sigma) and allowed to recover overnight. The next day, the media was removed and replaced with media supplemented with either 0.25 µM dexamethasone (Sigma), 2 mM L-arginine (Sigma), 10 mM L-citrulline (Sigma) or 0.25 µM histamine (Sigma). Cells were treated in triplicate for each stimulus and grown for 24 hours in supplemented media

### *RNA Isolation*

RNA was isolated using RNAqueous (Ambion) per the manufacturer's instructions. Samples were treated with 1 µL of DNase and 1/10<sup>th</sup> volume of DNase I Buffer for 20 minutes at 37°C to ensure removal of genomic DNA. The quantity of RNA was determined using spectrophotometry.

### *cDNA Preparation*

cDNA was prepared using the High Capacity Reverse Transcription Kit (Applied Biosystems). cDNA was synthesized in a 20-µl mixture containing 1 µg total RNA. Reverse transcription conditions were performed as suggested by the manufacturer for 40 cycles of 10 min at 25 °C, 120 min at 37 °C and 5 s at 85 °C. A negative control consisted of a samples lacking reverse transcriptase.

### *Primers and Probes*

qRT-PCR was performed using the following Taqman Gene Expression Assays ordered from Applied Biosystems: CPSI (Hs00919483\_m1), NAGs (Hs00400246\_m1), OTC (Hs00166892\_m1), ASS (Hs00540723\_m1), ASL (Hs00163695\_m1), ARG1 (Hs00968979\_m1), ARG2 (Hs00265750\_m1) eNOS (Hs00167166\_m1), iNOS (Hs00167257\_m1), nNOS (Hs00167223\_m1), GAPDH (4333764F), β-actin (ACTB) (4333762F).

### *Relative Expression of mRNA Transcripts by qRT-PCR*

qRT-PCR analyses for CPSI, NAGs, OTC, ASS, ASL, ARG1, ARG2, eNOS, iNOS, nNOS, GAPDH and β-actin mRNAs were performed using the ABI PRISM

7700 Sequence Detection System and its analysis software, SDS 2.3 and RQ Manager (PE Applied Biosystems, Inc., Foster City, CA). PCR was performed with TaqMan Universal PCR Master Mix (Applied Biosystems) using 3  $\mu$ L of diluted (1:3) cDNA, 200 nmol/L of probe, and 300 nmol/L of primers in a 10- $\mu$ L final reaction mixture. Each of the 50 PCR cycles consisted of 15 s of denaturation at 95 °C and hybridization of probe and primers for 1 min at 60 °C. Each reaction was carried out in triplicate using the same RNA source in at least three separate reverse transcriptase reactions for quality control and redundancy.

#### *Western Blotting and Densitometry*

Protein was isolated from cell cultures using Mammalian Protease Extraction Reagent (Pierce) supplemented with PMSF (Sigma) and Protease Inhibitor Cocktail (Sigma). Protein concentration was determined using the BCA Protein Assay Kit (Pierce). 20  $\mu$ g of protein was loaded onto a 4-20% Tris-HCl Ready Gel (Bio-Rad) and separated by electrophoresis at room temperature at 200 volts for 45 minutes. Protein was transferred to 0.45  $\mu$ M nitrocellulose membranes (Bio-Rad) for 1 hour at 100 volts at 4°C. Membranes were blocked in 5% milk in TBS-T for 1 hour at room temperature and then probed with 1:1000 of appropriate primary antibody diluted in 5% milk in TBS-T overnight at 4°C. The membranes were washed 3 times with 0.5% TBS-T for 10 minutes and then incubated in 1:5000 dilution of secondary antibody diluted in 5% milk in TBS-T for 2 hours at room temperature. The membranes were washed 3 times with 0.5% TBS-T for 10 minutes. Proteins were visualized using ECL Western Blotting Substrate (Pierce). Each membrane was exposed to Kodak Bio-Max film (PerkinElmer) and developed. Scanning

densitometry was calculated using TINA software (Raytest, Straubenhardt, Germany).

#### *Amino Acid Analysis*

Amino acids were separated by cation-exchange chromatography (7300 amino acid analyzer, Beckmann, Palo Alto, Calif.). Levels of all detectable amino acids were measured after calibration of the analyzer. Data are expressed as  $\mu\text{Moles}$  of Amino Acid / Total Protein ( $\mu\text{g}/\mu\text{L}$ ).

#### *Nitric Oxide Analysis*

Analysis of nitric oxide production via the measurement of nitric oxide metabolites ( $\text{NO}_x$ ) was performed on a Sievers 280i Nitric Oxide analyzer. Nitric oxide is measured by nitrites and nitrates. Data are expressed as  $\mu\text{Moles NO}_x$  / Total Protein ( $\mu\text{g}/\mu\text{L}$ ).

#### *Statistical Analysis*

Data is expressed as the average  $\pm$  standard deviation. Data was analyzed using unpaired t-test on GraphPad Software. P-values less than 0.05 are considered statistically significant.

## Results and Discussion

### *MRC5 Cells*

MRC-5 cells are derived from lung fibroblast tissue, developed in 1966 (Coriell). The karyotype of this cell line is 46, XY; normal diploid male. The cumulative population doubling time to senescence is 42 – 48 doublings. This cell line was utilized because it was thought to be a good vascular cell line to study ASS and ASL in the context of NOS enzymes. mRNA analysis reveals that this cell line expresses CPSI, NAGs, ASS, ASL and ARG2 (Figure 5.1). We did not detect OTC, ARG1 or expression of eNOS, iNOS or nNOS in this cell line (Figure 5.1). Enzyme transcript expression did not change significantly according to the t-tests in response to treatment with 0.25  $\mu$ M dexamethasone, 2 mM L-arginine, 10 mM L-citrulline or 10  $\mu$ M of histamine (Figure 5.1). Subsequent analysis of the intracellular levels of arginine and citrulline revealed low levels of arginine that were increased with 0.25  $\mu$ M dexamethasone treatment (Figure 5.2). It is interesting to note that citrulline was not detected in this cell line, until the cell line was stimulated with the addition of 10 mM L-citrulline (Figure 5.2). Citrulline was undetected in the control sample and increased to over 60  $\mu$ Moles/protein in the citrulline treated sample (Figure 5.2). Analysis of nitric oxide metabolites revealed moderate amounts that were increased by the treatment of the cell line with 10 mM L-citrulline (Figure 5.3). Western blot analysis was not conducted on this cell line and this cell line was not used for downstream analysis given its lack of expression of key urea cycle and nitric oxide cycle enzymes.

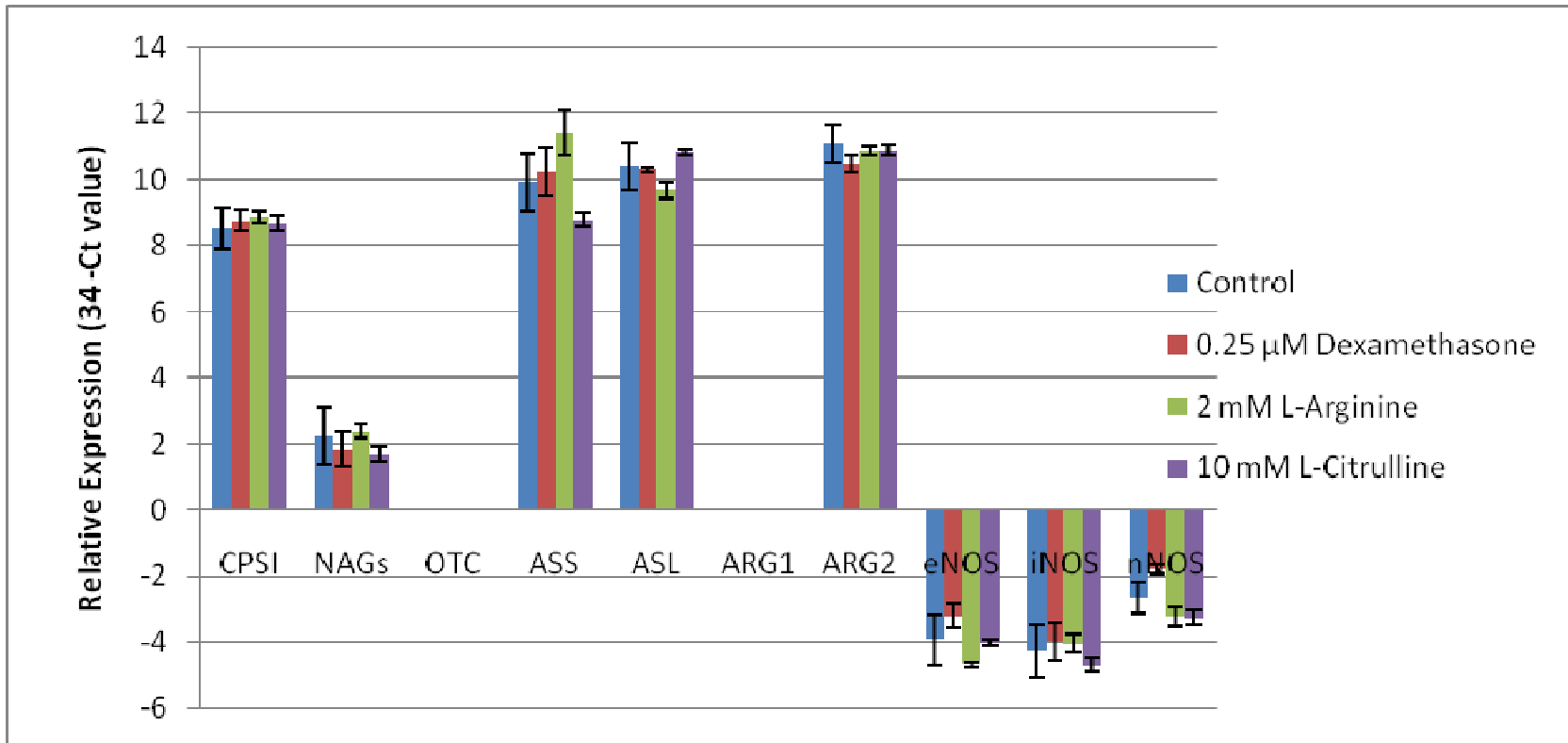


Figure 5.1 Raw Gene Expression Changes in MRC-5 Cells. Relative expression of qRT-PCR data was calculated using 34 – Ct method. OTC, eNOS, iNOS and nNOS were not detected in MRC5 cell samples. Experiments were repeated in triplicate.

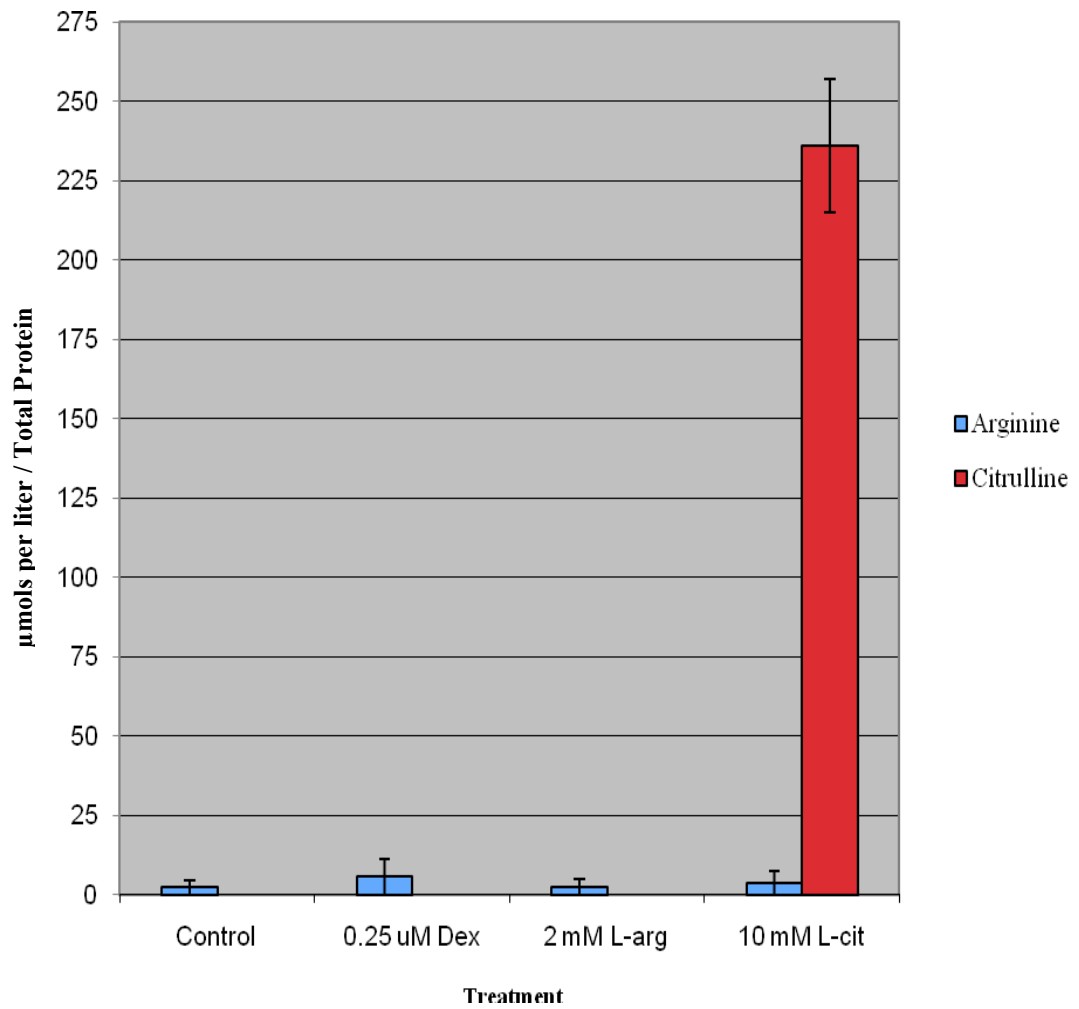


Figure 5.2 Intracellular Amino Acid Concentrations in MRC5 Cells. Citrulline was not detected in MRC5 samples except in sample treated with 10 mM L-citrulline. Experiments were repeated in triplicate and are expressed as the average  $\pm$  the standard deviation. \* indicates p-value  $<0.05$ .



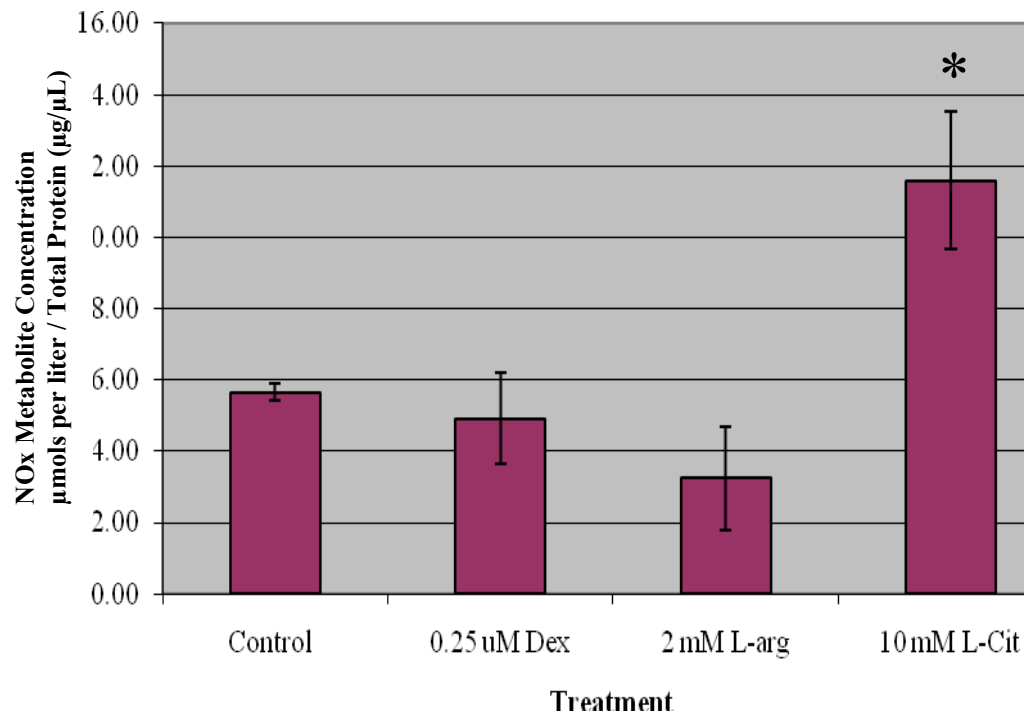


Figure 5.3 Intracellular Nitric Oxide Metabolite Concentrations in MRC5 Cells Data. Experiments were repeated in triplicate and are expressed as the average  $\pm$  the standard deviation.

### *HEPG2 Liver Cells*

HEPG2 liver cells are derived from a human hepatocellular cell line that was introduced in 1979 (Aden, Fogel, Plotkin, Damjanov, and Knowles, 1979). This cell line has been found to express a wide variety of liver specific functions and has been studied as a model for urea cycle enzymes, liver metabolism and development and oncogenesis (Javitt, 1990). The metabolic pathways that have been studied appear to simulate the normal behaviors of hepatocytes (Javitt, 1990). Initial gene expression studies detected mRNA expression of the complete complement of urea cycle enzymes as well as eNOS and iNOS (Figure 4.4). Additionally, the mRNA expression levels were similar between the HEPG2 cells (Figure 4.4) and the liver tissue mRNA from Chapter 3 (Figure 3.2).

### *Changes in mRNA and Protein Expression in HEPG2 Liver Cells*

Experiments show that HEPG2 cells express the full complement of urea cycle mRNAs transcripts. Additionally, these transcripts were affected by treatment of the media. For example, CPSI mRNA expression was significantly increased with 2 mM L-arginine ( $p=0.003$ ), 10 mM L-citrulline ( $p=0.0111$ ) and 10  $\mu$ M histamine ( $p<0.0001$ ). Although not statistically significant, CPSI mRNA expression was increased with 0.25  $\mu$ M dexamethasone treatment ( $p=0.1001$ ). NAGs mRNA expression was significantly decreased with 2 mM L-arginine ( $p=0.0132$ ) and increased with 10  $\mu$ M histamine ( $p=0.0123$ ). Although not statistically significant, NAGs mRNA expression was decreased with 10 mM L-citrulline ( $p=0.1568$ ) and increased with 0.25  $\mu$ M dexamethasone treatment ( $p=0.1129$ ). OTC mRNA

expression was significantly increased with 2 mM L-arginine ( $p=0.05$ ), 10 mM L-citrulline ( $p=0.05$ ) and 10  $\mu$ M histamine ( $p=0.0102$ ). Although not statistically significant, OTC mRNA expression was decreased with 0.25  $\mu$ M dexamethasone treatment ( $p=0.4444$ ). ASS mRNA expression did not vary within our experiments. Treatment with 0.25  $\mu$ M dexamethasone, 2 mM L-arginine, 10 mM L-citrulline and 10  $\mu$ M histamine did not significantly increase or decrease the mRNA expression of ASS. Western blot analysis indicated a significant increase in ASS protein expression with treatment of 0.25  $\mu$ M dexamethasone ( $p=0.0253$ ) despite insignificant changes in mRNA expression. The discordance observed between mRNA expression and protein expression indicates that ASS may be regulated in a complex manner by post-translational modifications. ASL mRNA expression was significantly increased with 2 mM L-arginine ( $p=0.05$ ), 10 mM L-citrulline ( $p=0.0365$ ) and 10  $\mu$ M histamine ( $p=0.002$ ). Although not statistically significant, OTC mRNA expression was increased with 0.25  $\mu$ M dexamethasone treatment ( $p=0.4444$ ). Western blot analysis indicated that ASL protein expression was increased insignificantly with 10 mM L-citrulline ( $p=0.0923$ ) and 10  $\mu$ M histamine ( $p=0.0985$ ). The Western Blot results varied between experiments, resulting in an abnormal averages and standard deviations. This antibody required significant optimization for experiments which may have contributed to the experimental variation in results. Additionally, the mRNA expression and protein expression for ASL was not concordant for any of the stimuli. ASL mRNA increased with all treatments, while the protein expression did not vary exceptionally. It is possible that post-transcriptional and pre-translational control mechanisms are involved in the

regulation of ASL protein expression. ARG1 mRNA expression was significantly increased with 0.25  $\mu$ M dexamethasone treatment ( $p=0.0208$ ), 2 mM L-arginine ( $p<0.0001$ ) and 10  $\mu$ M histamine ( $p<0.0001$ ). Although not statistically significant, ARG1 mRNA expression was increased by 10 mM L-citrulline ( $p=0.1102$ ). ARG1 protein expression did not vary significantly with changes in the extracellular stimuli. ARG1 protein expression did not correlate with mRNA expression. The ARG1 antibody was not robust and this may have led to variations in the experimental results. ARG2 mRNA expression was significantly increased with 10  $\mu$ M histamine ( $p<0.0006$ ). 0.25  $\mu$ M dexamethasone ( $p=0.2364$ ), 2 mM L-arginine ( $p=0.5282$ ) and 10 mM L-citrulline ( $p=0.3442$ ) had little effect on the ARG2 mRNA expression. eNOS mRNA expression was significantly increased with 10 mM L-citrulline ( $p=0.0403$ ) and significantly decreased with 0.25  $\mu$ M dexamethasone ( $p=0.0269$ ). 2 mM L-arginine ( $p=0.8488$ ) and 10  $\mu$ M histamine ( $p=0.5732$ ) had insignificant effects on eNOS mRNA expression. eNOS protein expression was not concordant with mRNA expression. eNOS protein expression decreased significantly with treatment of 10  $\mu$ M histamine ( $p=0.0199$ ) although this had little effect on the mRNA expression of eNOS. In endothelial cells, histamine has been reported to upregulate eNOS expression (Li, Burkhardt, Heinrich, Brausch, Xia, and Forstermann, 2003). Our studies indicate that this does not hold true for HEGP2 hepatoma cells. This discordance may be the result of tissue specific eNOS regulation. iNOS mRNA expression was detected at very low amounts in the control mRNA. The 34-Ct value for the control, 0.25  $\mu$ M dexamethasone and 2 mM L-arginine samples were less than 0 which indicates that mRNA was not detected until

the 34<sup>th</sup> cycle of PCR amplification, which means there is little iNOS RNA present and detected. However, treatment with 10 mM L-citrulline (p=0.0112) and 10  $\mu$ M histamine (p=0.0007) significantly increased transcript levels to a detectable level. HSP90 has been shown to activate eNOS (Aschner, Foster, Kaplowitz, Zhang, Zeng, and Fike, 2007; Aschner, Zeng, Kaplowitz, Zhang, Slaughter, and Fike, 2009; Chen and Meyrick, 2004). Although we did not measure HSP90 mRNA expression we found that 0.25  $\mu$ M dexamethasone (p=0.1470), 2 mM L-arginine (p=0.959) increased HSP90 protein expression but not significantly. Additionally, 10 mM L-citrulline decreased HSP90 protein expression (p=0.0979), although this change was not significant. Most interesting, 10  $\mu$ M of histamine significantly decreased HSP90 (p=0.039). This decrease in protein expression of HSP90 correlates with a significant decrease in eNOS protein level (p=0.0007). This result, in addition to the literature, suggests that HSP90 may play an important role in the regulation of endothelial nitric oxide production.

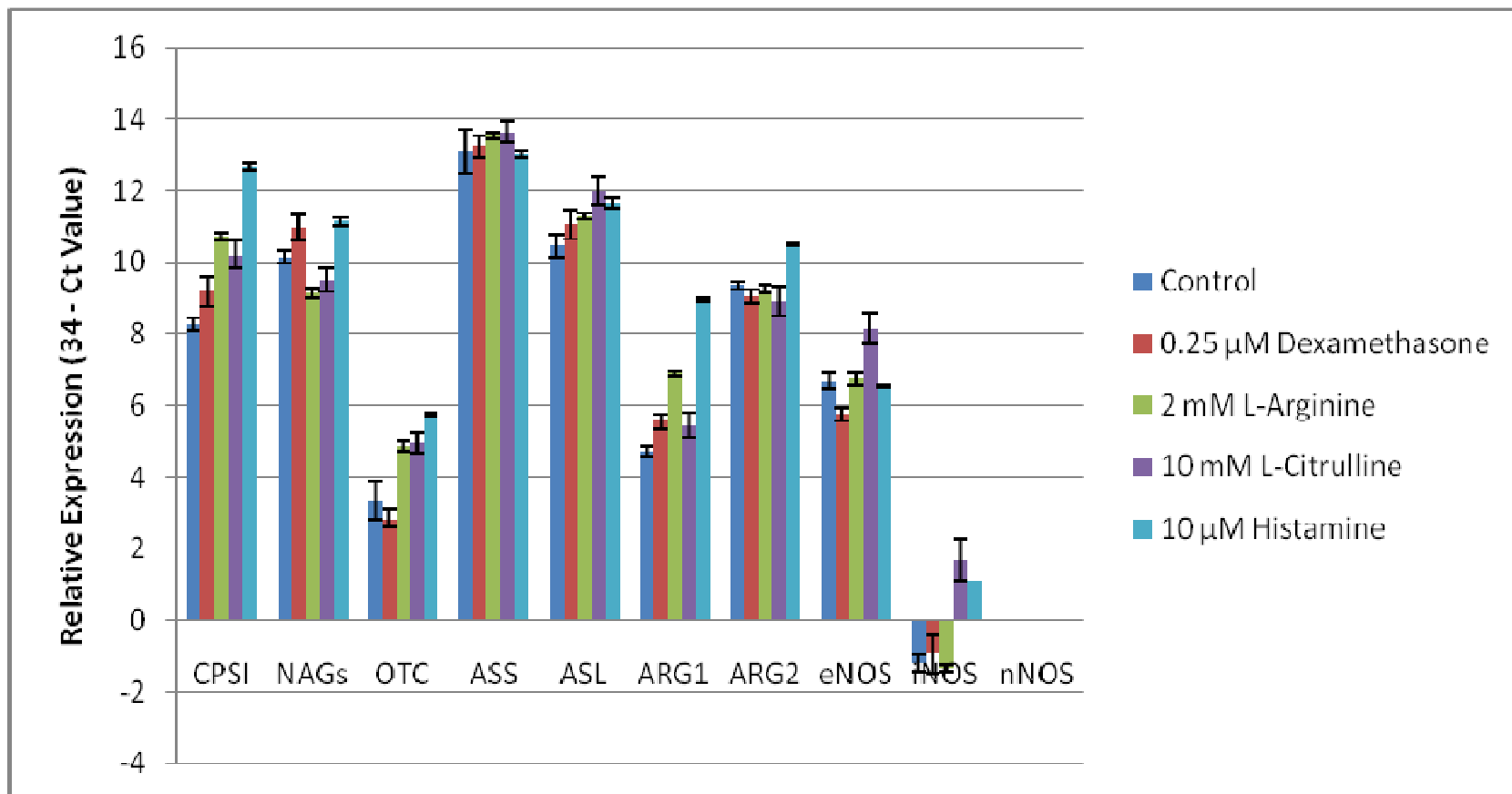


Figure 5.4 Raw Changes in Gene Expression in HEPG2 Cells. Relative expression of qRT-PCR data was calculated using 34 – Ct method. nNOS was not detected in HEPG2 cell samples. Experiments were repeated in triplicate and are expressed as the average  $\pm$  the standard deviation.

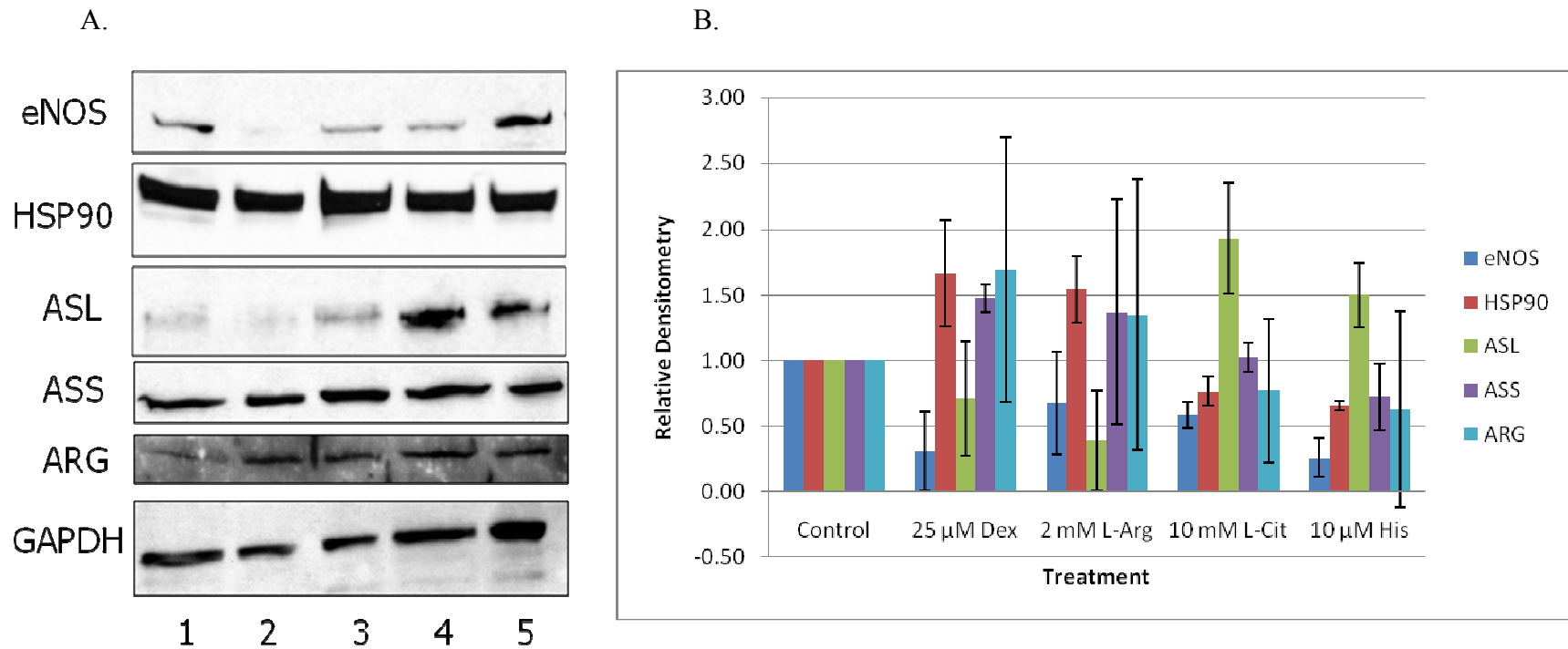


Figure 5.5 Changes in Protein Expression in HEPG2 Cells. Panel A. Representative Western Blot Raw data using 20  $\mu$ g HEPG2 cell protein. Lane 1 Control cell lysate; Lane 2 cells treated with 0.25  $\mu$ M dexamethasone; Lane 3 cells treated with 2mM L-arginine; Lane 4 cells treated with 10 mM L-citrulline; Lane 5 cells treated with 10  $\mu$ M histamine. Experiments were repeated in triplicate. Band density was normalized to GAPDH expression using densitometry. Data in Panel B are expressed as average densitometry  $\pm$  standard deviation (SD).

*Changes in Amino Acid and Nitric Oxide Metabolite Concentrations in HEPG2 Liver Cells*

*Citrulline*

Levels of intracellular and extracellular arginine and citrulline which are the intermediates involved in both the urea and nitric oxide cycles were measured. We also measured intracellular and extracellular ornithine concentrations because ornithine is a by-product of urea formation through the enzyme ARG1 and is recycled to the mitochondria where it utilized as substrate by OTC. Extracellular citrulline did not change significantly due to treatment with 0.25  $\mu$ M dexamethasone ( $p=0.7940$ ). Extracellular citrulline concentration increased significantly with 10 mM L-citrulline treatment ( $p<0.0001$ ), which would be expected because we were supplementing the medium with citrulline and increased not significantly with 2 mM L-arginine ( $p=0.828$ ). Interestingly, extracellular citrulline decreased with treatment of 10  $\mu$ M histamine ( $p=0.0435$ ). Histamine has been shown to increase the production of nitric oxide; we propose that the extracellular citrulline was transported into the cellular milieu for nitric oxide production.

Intracellular levels of citrulline were close to the extracellular levels in control cells. Intracellular citrulline did not change with the addition of 0.25  $\mu$ M dexamethasone to the extracellular medium ( $p=0.2483$ ). 2 mM L-arginine ( $p=<0.001$ ) and 10 mM L-citrulline ( $p<0.001$ ) significantly increased levels of intracellular arginine. We are not certain why arginine would increase intracellular citrulline. It is possible that some of the arginine is being used for nitric oxide production and the increase in citrulline is a by-product of increased nitric oxide



production. It is interesting that 10  $\mu$ M histamine significantly decreased extracellular levels of citrulline, yet intracellular levels of citrulline remain mostly unchanged ( $p=0.2816$ ).

### *Ornithine*

Extracellular ornithine concentrations did not change significantly in response to 0.25  $\mu$ M dexamethasone ( $p=0.4221$ ), 2 mM L-arginine ( $p=0.3523$ ) and 10 mM L-citrulline ( $p=0.5702$ ). Extracellular ornithine concentration decreased significantly with 10  $\mu$ M histamine ( $p=0.0121$ ). Our hypothesis is that histamine increases nitric oxide production, and ornithine enters the cell in order to be recycled for substrate for OTC. Intracellular ornithine concentrations were significantly increased by 2 mM L-arginine ( $p=0.0084$ ) and 10 mM L-citrulline ( $p=0.0093$ ).

### *Arginine*

Extracellular arginine concentration was increased with 2 mM L-arginine ( $p<0.0001$ ), 10 mM L-citrulline ( $p=0.0205$ ) and 10  $\mu$ M histamine ( $p=0.0006$ ). It is possible that the addition of citrulline to the extracellular medium causes the cell to produce and export arginine. Intracellular arginine was not detected until the cells were treated with 2 mM L-arginine.

### *Nitric Oxide Metabolites*

Extracellular nitric oxide metabolites were increased with 0.25  $\mu$ M dexamethasone ( $p=0.0021$ ), 2 mM L-arginine ( $p=0.0006$ ), 10 mM L-citrulline ( $p=0.0043$ ) and 10  $\mu$ M histamine ( $p=0.0001$ ).

Intracellular nitric oxide metabolites were increased significantly with 10 mM L-citrulline ( $p=0.0102$ ), indicating an increase in nitric oxide production.

Intracellular nitric oxide metabolites were significantly decreased with 2 mM L-arginine ( $p=0.0046$ ) and 10  $\mu$ M L-histamine ( $p=0.0045$ ).

Sample Name	Citrulline ( $\mu\text{M}$ )	Ornithine ( $\mu\text{M}$ )	Arginine ( $\mu\text{M}$ )
Control Cell Media	23 $\pm$ 11.49	247.79 $\pm$ 95.29	353.07 $\pm$ 77.06
0.25 $\mu\text{M}$ Dex Cell Media	20.93 $\pm$ 5.75	196.39 $\pm$ 29.09	413.43 $\pm$ 92.84
2 mM L-Arg Cell Media	49.29 $\pm$ 16.08	331.97 $\pm$ 100.72	4355.33 $\pm$ 77.14
10 mM L-Cit Cell Media	10473.93 $\pm$ 2705.67	210.96 $\pm$ 39.84	572.04 $\pm$ 66.74
10 $\mu\text{M}$ His Cell Media	3.43 $\pm$ 1.80	8.09 $\pm$ 3.55	905.58 $\pm$ 57.26

Table 5.1 Extracellular Concentrations of Amino Acids in HEPG2 Cells. Experiments were repeated in triplicate and are expressed as the average  $\pm$  the standard deviation.

Sample Name	Citrulline ( $\mu\text{M}$ )	Ornithine ( $\mu\text{M}$ )	Arginine ( $\mu\text{M}$ )
Control Cell Lysate	20.96 $\pm$ 3.76	5.53 $\pm$ 2.65	Not Detected
0.25 $\mu\text{M}$ Dex Cell Lysate	24.98 $\pm$ 3.53	3.69 $\pm$ 3.30	Not Detected
2 mM L-Arg Cell Lysate	220.55 $\pm$ 4.09	19.98 $\pm$ 4.44	25.26 $\pm$ 3.13
10 mM L-Cit Cell Lysate	1179.20 $\pm$ 45.83	14.50 $\pm$ 1.97	Not Detected
10 $\mu\text{M}$ His Cell Lysate	23.8 $\pm$ 1.23	2.45 $\pm$ 1.22	Not Detected

Table 5.2 Intracellular Concentrations of Amino Acids in HEPG2 Cells. Experiments were repeated in triplicate and are expressed as the average  $\pm$  the standard deviation.

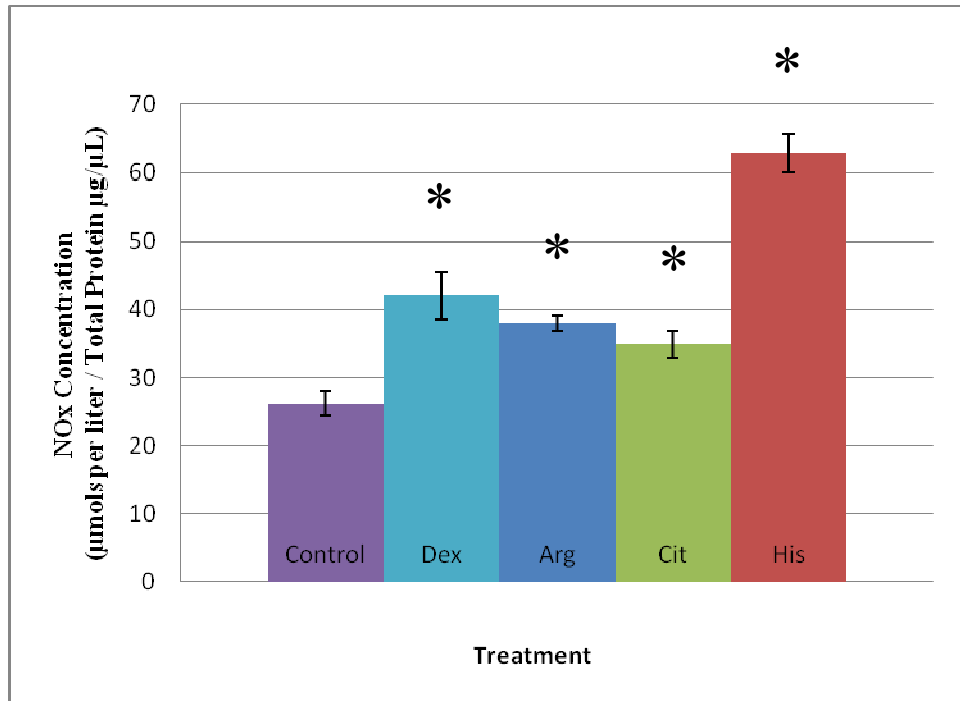


Figure 5.6 Changes in Extracellular Nitric Oxide Metabolites in HEPG2 Cells. Experiments were repeated in triplicate and are expressed as the average  $\pm$  the standard deviation. \* indicates p-value  $< 0.05$ .

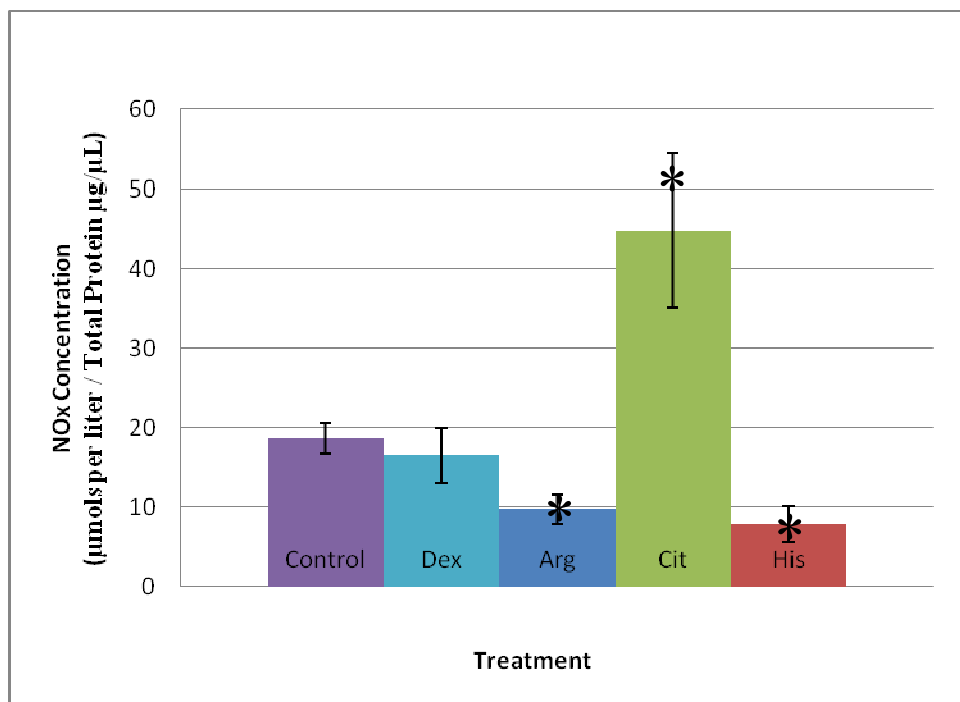


Figure 5.7 Changes in Intracellular Nitric Oxide Metabolites in HEPG2 Cells. Experiments were repeated in triplicate and are expressed as the average  $\pm$  the standard deviation. \* indicates p-value  $< 0.05$ .

### *Summary of Results for HEPG2 Liver Cells*

HEPG2 cells expressed mRNA for all of the urea cycle enzymes and eNOS. iNOS was induced with 10 mM L-citrulline and 10  $\mu$ M histamine. When comparing the mRNA expression with the protein expression we noticed that most of the results were discordant indicating that these enzymes (ASS, ASL, ARG1, eNOS) are likely regulated in a complex manner at the post-translational level. Furthermore, citrulline treatment increased the production of intracellular nitric oxide metabolites and extracellular nitric oxide metabolites as well as eNOS and iNOS mRNA transcripts though it did not change eNOS protein expression. 10  $\mu$ M histamine had the different effect, increasing extracellular levels of nitric oxide metabolites and decreasing intracellular levels of nitric oxide metabolites. It is possible that nitric oxide was produced in the cell and then transported into the extracellular milieu where the concentration of histamine was highest.

### *HEK Kidney Cells*

HEK kidney cells are adherent cells derived from human embryonic kidney in 1977 (Graham, Smiley, Russell, and Nairn, 1977). This cell line was generated by transformation with sheared adenovirus 5 DNA (Graham *et al.*, 1977). Results from mRNA expression analysis reveal that the HEK kidney cells express the same urea and nitric oxide cycle enzymes as our human tissue studies (Figure 4.4). Neither this cell line, nor the human kidney tissue produced ARG1 that we could detect using quantitative real-time PCR (qRT-PCR). This is consistent with the kidney's known biological role of endogenous arginine production (Dhanakoti, Brosnan, Herzberg, and Brosnan, 1990; Dhanakoti, Brosnan, Herzberg, and Brosnan, 1992; Wu *et al.*, 1998). The HEK cell line had minimal expression of NAGs, OTC, eNOS and iNOS – although stimuli were able to induce iNOS expression (Figure 4.5).

### *Changes in mRNA and Protein Expression in HEK Kidney Cells*

CPSI mRNA expression was decreased, although not significantly, with 0.25  $\mu$ M dexamethasone ( $p=0.0933$ ). There was variation in the control sample which may have affected the significance of the effects induction of mRNA expression of CPSI by 2 mM L-arginine ( $p=0.1920$ ). We did not detect NAGs, OTC, ARG1 or eNOS mRNA expression in HEK cells. ASS mRNA expression did not vary significantly within our experiments for 0.25  $\mu$ M dexamethasone ( $p=0.1183$ ), 2 mM L-arginine ( $p=1.0$ ) and 10 mM L-citrulline ( $p=0.1778$ ). Treatment with 10  $\mu$ M histamine significantly increased the mRNA expression of ASS ( $p=0.202$ ). Western blot analysis indicated a no significant variations in ASS protein expression for 25

$\mu\text{M}$  dexamethasone ( $p=0.0253$ ), 2 mM L-arginine ( $p=0.1258$ ), 10 mM L-citrulline ( $p=0.7304$ ) and 10  $\mu\text{M}$  histamine ( $p=0.6272$ ). The large standard deviation in ASS protein expression in 10  $\mu\text{M}$  histamine treated cells indicates that there might be experiment based variation. The discordance between ASS mRNA expression and protein expression for 10  $\mu\text{M}$  histamine treated cells suggests that the regulation of ASS is complex and might involve post-translational modifications and/or interactions with other proteins or enzymes. ASL mRNA expression did not vary significantly within our experiments for 0.25  $\mu\text{M}$  dexamethasone ( $p=0.2691$ ), 2 mM L-arginine ( $p=0.1479$ ) and 10 mM L-citrulline ( $p=0.3089$ ) or with 10  $\mu\text{M}$  histamine ( $p=0.1971$ ). Western blot analysis indicated no significant variations in ASL protein expression for 25  $\mu\text{M}$  dexamethasone ( $p=0.5734$ ), 2 mM L-arginine ( $p=0.5386$ ), 10 mM L-citrulline ( $p=0.5200$ ) and 10  $\mu\text{M}$  histamine ( $p=0.5031$ ). The large standard deviation in ASL protein expression made it difficult to analyze the data critically. This is a result of an ineffective antibody and experimental variation. However, the mRNA and protein expression is concordant for ASL in this cell line. ARG2 mRNA expression did not vary significantly with 0.25  $\mu\text{M}$  dexamethasone ( $p=0.2310$ ), 2 mM L-arginine ( $p=0.6717$ ), 10 mM L-citrulline  $p=0.7836$ ) and 10  $\mu\text{M}$  histamine ( $p=0.0871$ ). iNOS mRNA expression was detected at very low amounts. The 34 minus Ct value for the control, 2 mM L-arginine and 10 mM L-citrulline samples were less than 0 which indicates that mRNA was not detected until after the 34<sup>th</sup> cycle of PCR amplification, which means there is little transcript present and detected. Treatment with 0.25  $\mu\text{M}$  dexamethasone insignificantly increased the relative value to 0.49. Interestingly, 10  $\mu\text{M}$  histamine ( $p<0.0001$ ) significantly

increased transcript levels to a relative value of 3.32. This supports studies in the literature showing that histamine induces the production of nitric oxide; however this is a novel finding in kidney cells (Li *et al.*, 2003;Mannaioni, Bello, Di Bello, Mirabella, Gai, Schunack, and Masini, 1997;Mannaioni, Bello, Di Bello, Schunack, and Masini, 1997;Tanimoto, Wang, Murata, Kimura, Nomaguchi, Nakata, Tsutsui, and Sasaguri, 2007).



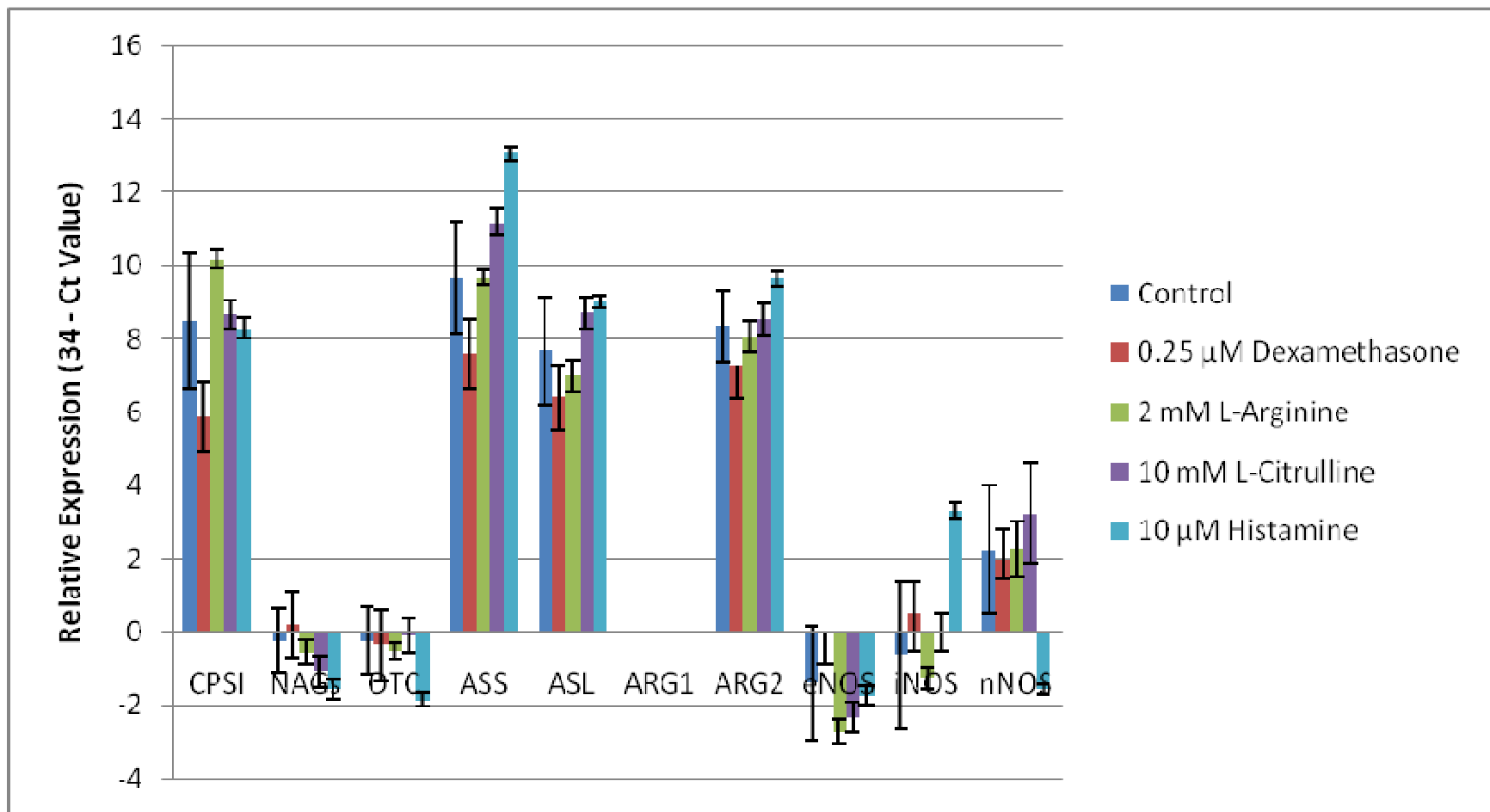
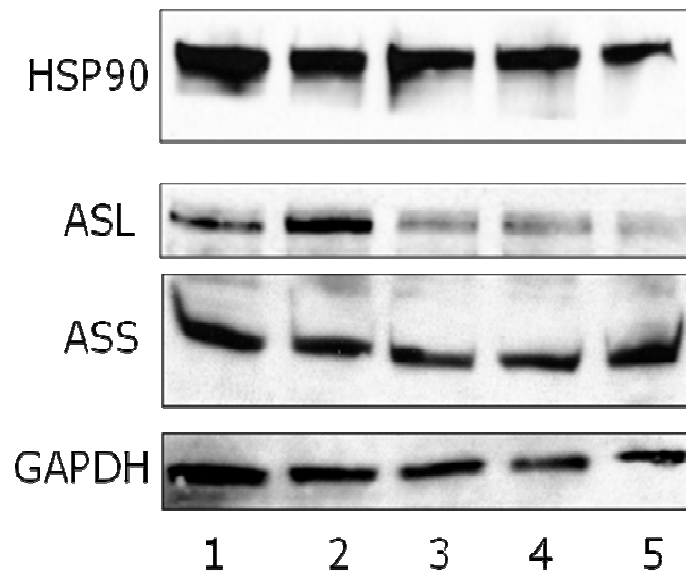


Figure 5.8 Raw Changes in Gene Expression HEK Cells. Experiments were repeated in triplicate and are expressed as the average  $\pm$  the standard deviation.

A.



B.

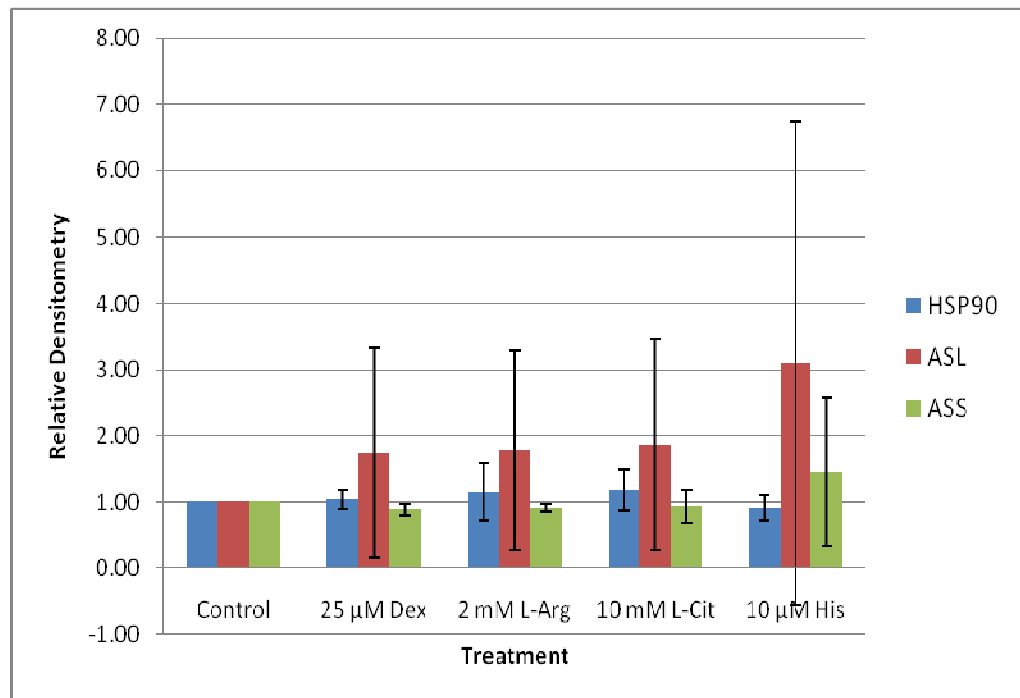


Figure 5.9 Changes in Protein Expression HEK Cells. Panel A. Representative Western Blot Raw data using 20 μg HEK cell protein. Lane 1 Control cell lysate; Lane 2 cells treated with 0.25 μM dexamethasone; Lane 3 cells treated with 2mM L-arginine; Lane 4 cells treated with 10 mM L-citrulline; Lane 5 cells treated with 10 μM histamine. Experiments were repeated in triplicate. Band density was normalized to GAPDH expression using densitometry. Data in Panel B are expressed as average densitometry ± standard deviation (SD).

*Changes in Amino Acid and Nitric Oxide Metabolite Concentrations in  
HEK Kidney Cells*

*Citrulline*

We measured levels of intracellular and extracellular arginine, citrulline and ornithine (Table 4.3). Extracellular citrulline was not detected until 2 mM L-arginine or 10 mM L-citrulline was added to the media. Intracellular levels of citrulline were similar to the extracellular levels in that it was not detected until 10 mM L-citrulline was added to the media.

*Ornithine*

Extracellular ornithine concentrations did not change significantly in response to 0.25  $\mu$ M dexamethasone ( $p=0.3969$ ) or 10  $\mu$ M histamine ( $p=0.3864$ ). It is interesting to note that ornithine concentrations increased significantly with 2 mM L-arginine treatment ( $p=0.012$ ) while ornithine concentrations decreased significantly with 10 mM L-citrulline treatment ( $p=0.0245$ ). This suggests that ornithine is being re-generated from arginine, although this cell line lacks the necessary ARG1 to perform this function. It is possible that ARG2 is able to step in to fulfill this role. Also, citrulline decreased ornithine concentration significantly, suggesting that this cell line preferentially uses citrulline for nitric oxide production. There may also be other biological processes involved which interact to generate ornithine in this cell type. Intracellular ornithine was not detected in control cells. Ornithine concentration increased significantly in the 2 mM L-arginine treated samples ( $p=0.0002$ ). Although 0.25  $\mu$ M dexamethasone and 10  $\mu$ M histamine also

increased ornithine concentration their p-values were not significant due to the variation in the samples.

### *Arginine*

Extracellular arginine concentrations increased significantly in response to treatment with 2 mM L-arginine (p=0.0199), 10 mM L-citrulline (p=0.0002) and 10  $\mu$ M histamine (p=0.0481). 0.25  $\mu$ M dexamethasone also increased extracellular arginine concentrations but not significantly (p=0.1227).

Intracellular arginine concentration increased insignificantly with 2 mM L-arginine (p=0.3878), 10 mM L-citrulline (p=0.1519) and decreased without significance by 0.25  $\mu$ M dexamethasone (p=0.2983) and 10  $\mu$ M histamine (p=0.8879).

### *Nitric Oxide Metabolites*

Extracellular nitric oxide metabolites were increased with 0.25  $\mu$ M dexamethasone (p=0.0106), 2 mM L-arginine (p=0.0071), 10 mM L-citrulline (p=0.0031) and 10  $\mu$ M histamine (p=0.0120). Intracellular nitric oxide metabolites were not significantly changed in response to 0.25  $\mu$ M dexamethasone (p=0.3690), 2 mM L-arginine (0.2072), 10 mM L-citrulline (0.8869) or 10  $\mu$ M histamine (p=0.5324).

Sample Name	Citrulline ( $\mu\text{M}$ )	Ornithine ( $\mu\text{M}$ )	Arginine ( $\mu\text{M}$ )
Control Media	0	25.31 $\pm$ 7.31	139.98 $\pm$ 34.77
0.25 $\mu\text{M}$ Dex Media	0	16.89 $\pm$ 12.48	206.74 $\pm$ 47.97
2 mM L-Arg Media	3.74 $\pm$ 2.25	562.75 $\pm$ 203.19	7086.33 $\pm$ 3204.51
10 mM L-Cit Cell Media	5409 $\pm$ 186.32	10.17 $\pm$ 1.44	544.35 $\pm$ 44.02
10 $\mu\text{M}$ His Cell Media	0	29.43 $\pm$ 0.74	278.09 $\pm$ 77.53

Table 5.3 Extracellular Changes in Amino Acids in HEK Cells. Experiments were repeated in triplicate and are expressed as the average  $\pm$  the standard deviation.

Sample Name	Citrulline ( $\mu\text{M}$ )	Ornithine ( $\mu\text{M}$ )	Arginine ( $\mu\text{M}$ )
Control Cell Lysate	0	0	10.72 $\pm$ 4.92
0.25 $\mu\text{M}$ Dex Cell Lysate	0	2.20 $\pm$ 3.33	6.08 $\pm$ 4.59
2 mM L-Arg Cell Lysate	0	5.68 $\pm$ 0.72	15.95 $\pm$ 7.96
10 mM L-Cit Cell Lysate	285.76 $\pm$ 6.94	0.24 $\pm$ 0.42	20.53 $\pm$ 8.26
10 $\mu\text{M}$ His Cell Lysate	0	5.66 $\pm$ 7.79	9.86 $\pm$ 8.61

Table 5.4 Intracellular Changes in Amino Acids in HEK Cells. Experiments were repeated in triplicate and are expressed as the average  $\pm$  the standard deviation.

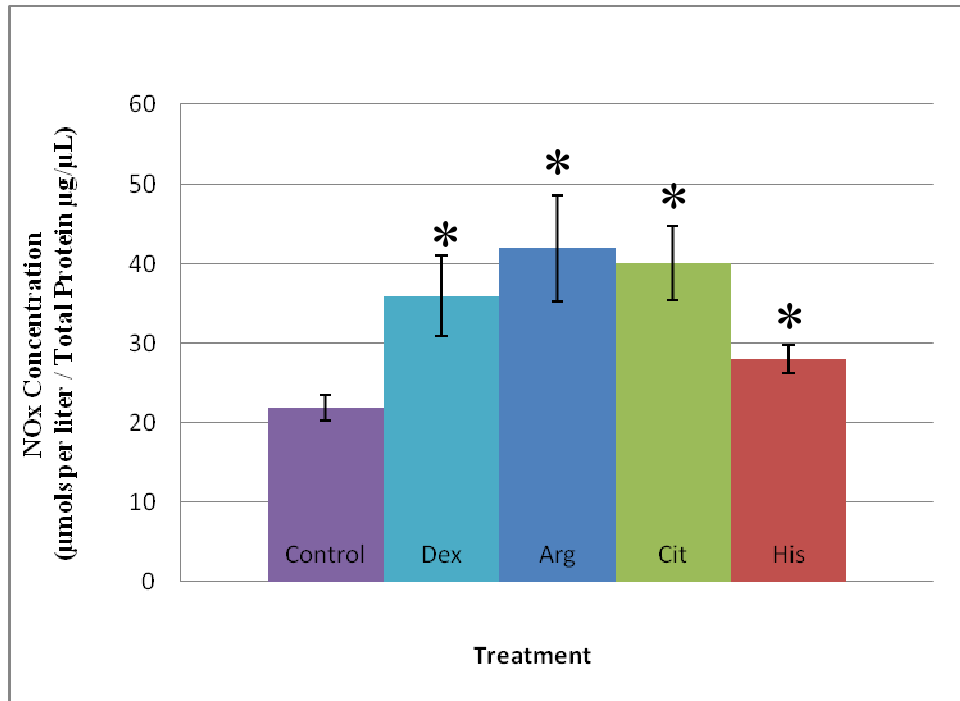


Figure 5.10 Changes in Extracellular Nitric Oxide Metabolites in HEK Cells. Experiments were repeated in triplicate and are expressed as the average  $\pm$  the standard deviation. \* indicates p-value  $< 0.05$ .

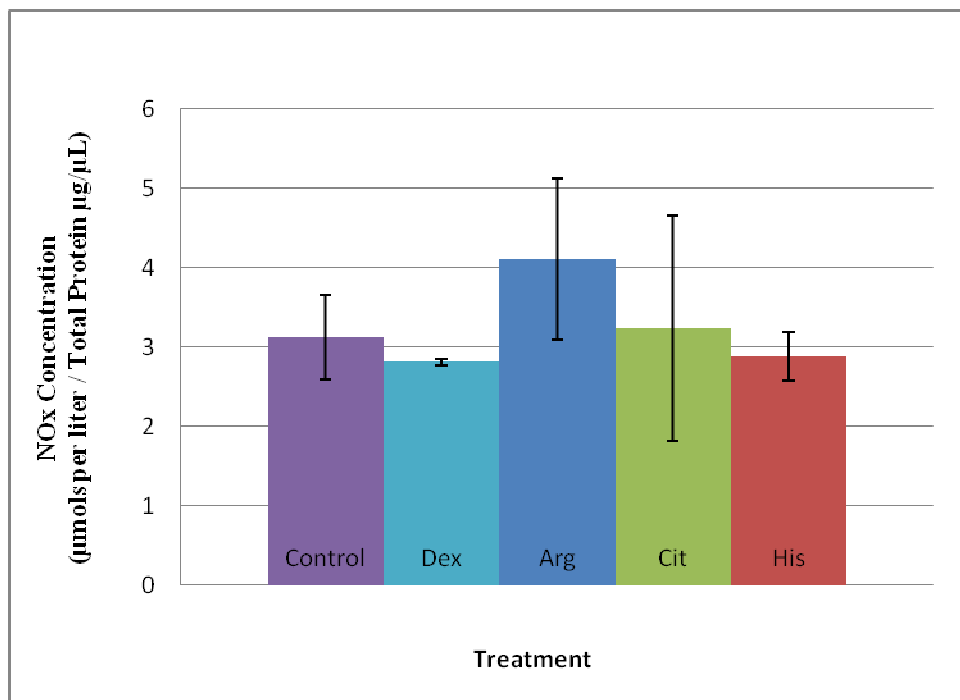


Figure 5.11 Changes in Intracellular Nitric Oxide Metabolites in HEK Cells. Experiments were repeated in triplicate and are expressed as the average  $\pm$  the standard deviation. \* indicates p-value  $< 0.05$ .

### *Summary of Results for HEK Kidney Cells*

HEK cells expressed mRNA for all of the enzymes tested except: NAGs, OTC, ARG1 and eNOS. The changes between mRNA and protein expression for ASS and ASL were more similar in the HEK cells than in the HEPG2 cells indicating that there might be tissue specific expression and regulation of these two enzymes. It is interesting that 2 mM arginine treatment increased citrulline concentration in the media and not inside the cell. It is also interesting that citrulline is not detected in either the medium or in the cell lysate except for in the citrulline treated medium. Given the large increase in concentration, we can determine that citrulline can easily enter and exit the cell. Treatment with 10 mM L-citrulline increased arginine concentration both in the media and inside the cell, indicating that potentially the expression of ASS and ASL proteins permitted the cell to make arginine using citrulline as substrate. Finally, nitric oxide metabolite concentrations did not change significantly in the cell lysate although significant increases were detected extracellular. It is possible that the nitric oxide was generated inside the cell and quickly exited the cell. Given the nature of the nitric oxide molecule, this is plausible.

### *HUVEC Endothelial Cells*

Human umbilical vein endothelial cells (HUVEC) are isolated from normal human umbilical vein. They are primary cells that are cryo-preserved at the end of primary culture and can be propagated for 16 population doublings before senescence. Endothelial cells play a pivotal role in a diverse range of physiological processes such as angiogenesis, arterial diseases and nitric oxide metabolism.

### *Changes in mRNA and Protein Expression in HUVEC Endothelial Cells*

HUVEC cells express a small amount of CPSI mRNA that did not vary with treatment of 0.25  $\mu$ M dexamethasone, 2 mM L-arginine, 10 mM L-citrulline or 10  $\mu$ M histamine. HUVEC cells express NAGs mRNA that did not vary with treatment of 0.25  $\mu$ M dexamethasone, 2 mM L-arginine, 10 mM L-citrulline or 10  $\mu$ M histamine. OTC mRNA expression was insignificantly detected in all samples. Given that 34 minus Ct make our cut-off for mRNA expression equal to 0, the relative values for OTC expression due to all treatments was negative. HUVEC cells express ASS mRNA that did not vary with treatment of 0.25  $\mu$ M dexamethasone, 2 mM L-arginine, 10 mM L-citrulline or 10  $\mu$ M histamine. Interestingly, ASS protein expression did increase significantly in response to 2 mM L-arginine treatment ( $p=0.0288$ ). Although treatment with 10  $\mu$ M histamine increased ASS protein expression, the difference from control was not significant due to the large standard deviation, indicating the influence of experimental variation on the result. HUVEC cells express ASL mRNA that did not vary with treatment of 0.25  $\mu$ M dexamethasone, 2 mM L-arginine, 10 mM L-citrulline or 10  $\mu$ M histamine. ASL



protein expression increased significantly in response to treatment with 0.25  $\mu$ M dexamethasone ( $p=0.0321$ ), 10 mM L-citrulline ( $p=0.0321$ ) and 10  $\mu$ M histamine ( $p=0.0214$ ). ARG1 mRNA expression was insignificantly detected in all samples. Given that 34 minus Ct make our cut-off for mRNA expression equal to 0, the relative values for ARG1 expression due to all treatments was negative. ARG1 protein was not detected in the HUVEC cell lysate using Western Blot Analysis. HUVEC cells express ARG2 mRNA that did not vary with treatment of 0.25  $\mu$ M dexamethasone, 2 mM L-arginine, 10 mM L-citrulline or 10  $\mu$ M histamine. HUVEC cells express eNOS mRNA that did not vary with treatment of 0.25  $\mu$ M dexamethasone, 2 mM L-arginine, 10 mM L-citrulline or 10  $\mu$ M histamine. eNOS protein was significantly increased with 2 mM L-arginine treatment. iNOS mRNA expression was not detected in the samples. Given that 34 minus Ct make our cut-off for mRNA expression equal to 0, the relative values for iNOS expression due to all treatments was negative. nNOS mRNA expression was insignificantly detected in all samples. Given that 34 minus Ct make our cut-off for mRNA expression equal to 0, the relative values for nNOS expression due to all treatments was negative. HSP90 protein increased with treatment of 0.25  $\mu$ M dexamethasone ( $p=0.0513$ ), 2 mM L-arginine ( $p=0.0855$ ) and 10 mM L-citrulline ( $p=0.0260$ ).

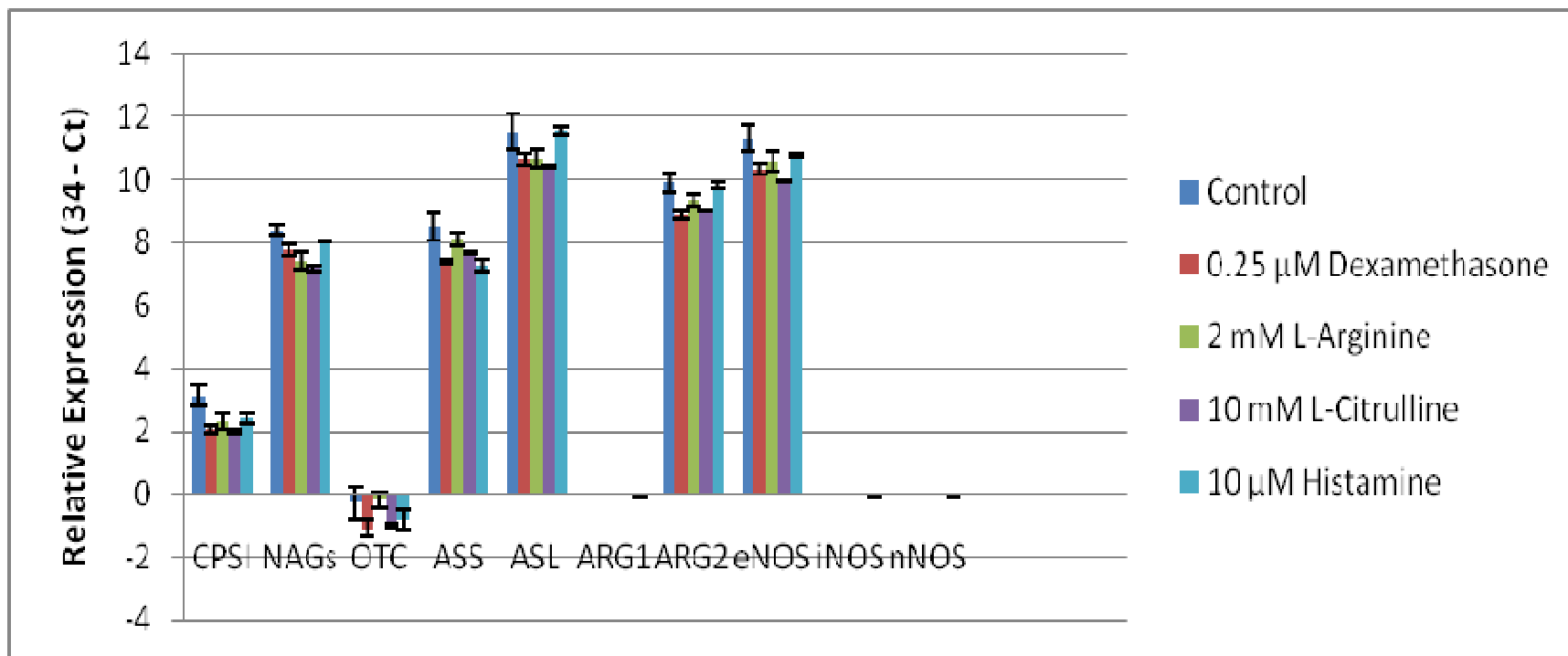


Figure 5.12 Raw Changes in Gene Expression in HUVEC Cells. Experiments were repeated in triplicate and are expressed as the average  $\pm$  the standard deviation.

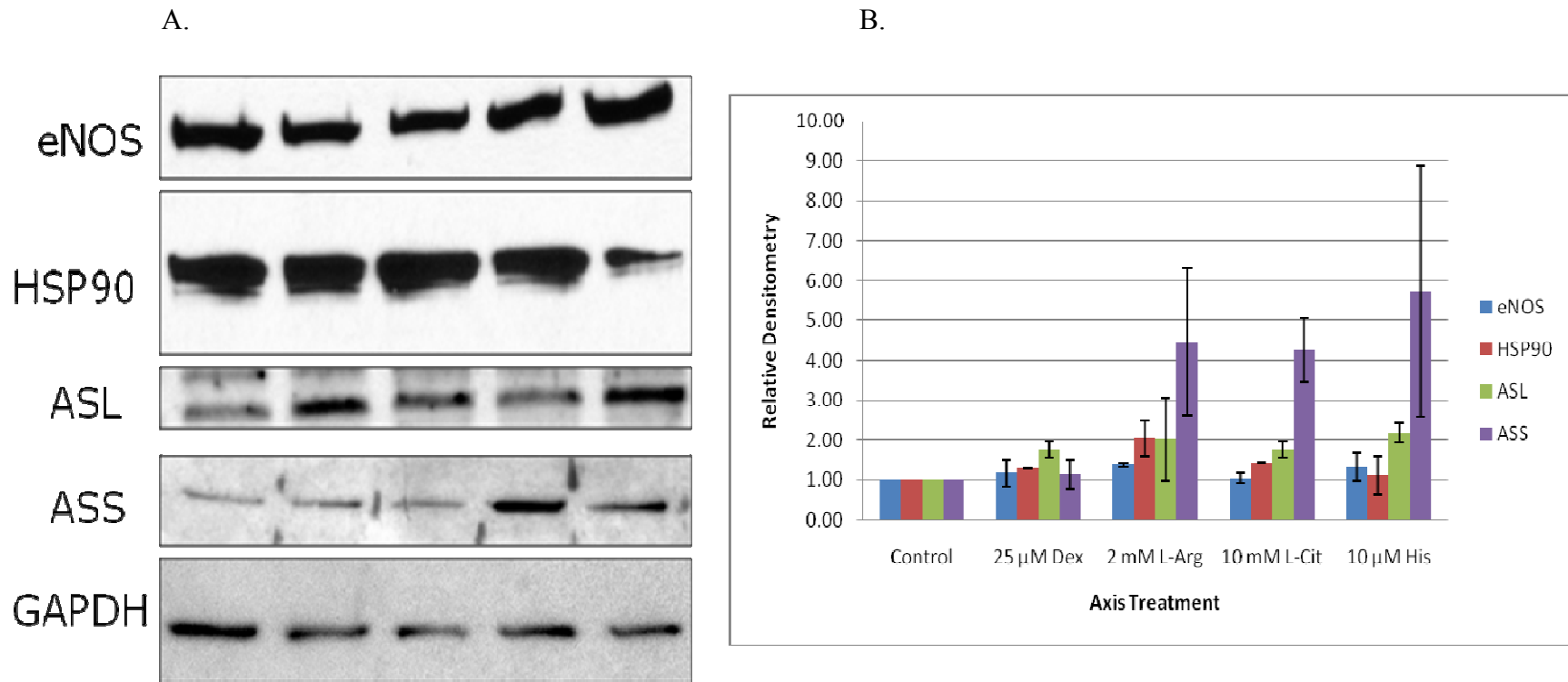


Figure 5.13 Changes in Protein Expression in HUVEC Cells. Panel A. Representative Western Blot Raw data using 20  $\mu$ g HUVEC cell protein. Lane 1 Control cell lysate; Lane 2 cells treated with 0.25  $\mu$ M dexamethasone; Lane 3 cells treated with 2mM L-arginine; Lane 4 cells treated with 10 mM L-citrulline; Lane 5 cells treated with 10  $\mu$ M histamine. Experiments were repeated in triplicate. Band density was normalized to GAPDH expression using densitometry. Data in Panel B are expressed as average densitometry  $\pm$  standard deviation (SD).

*Changes in Amino Acid and Nitric Oxide Metabolite Concentrations in HUVEC Endothelial Cells*

*Citrulline*

We measured levels of intracellular and extracellular arginine, citrulline and ornithine (Table 4.3). Extracellular citrulline was not detected in the control sample, the arginine treated sample or the histamine treated sample. 0.25  $\mu$ M dexamethasone increased extracellular citrulline concentration ( $p=0.0168$ ) as did 10 mM L-citrulline ( $p=0.0002$ ). Intracellular levels of citrulline were similar to the extracellular levels in that citrulline was not detected in samples except for the citrulline treated sample ( $p=0.0004$ ).

*Ornithine*

Extracellular ornithine concentrations increased significantly in response to 0.25  $\mu$ M dexamethasone ( $p=0.02$ ) and 2 mM L-arginine ( $p=0.0190$ ). Ornithine was not detected in the samples treated with 10  $\mu$ M histamine. Intracellular ornithine was not detected in any of the samples.

*Arginine*

Extracellular arginine concentrations increased significantly in response to treatment with 0.25  $\mu$ M dexamethasone ( $p=0.003$ ), 2 mM L-arginine ( $p<0.0001$ ) and 10  $\mu$ M histamine ( $p=0.0513$ ). Intracellular arginine concentration increased significantly only with 2 mM L-arginine ( $p=0.0014$ ). 0.25  $\mu$ M dexamethasone decreased intracellular arginine concentration but not with significance ( $p=0.2185$ ).

Both 10 mM L-citrulline and 10  $\mu$ M histamine had little effect on intracellular arginine concentration.

#### *Nitric Oxide Metabolites*

Extracellular nitric oxide metabolites were significantly increased with 0.25  $\mu$ M dexamethasone ( $p < 0.0001$ ), 2 mM L-arginine ( $p = 0.0050$ ) and 10 mM L-citrulline ( $p = 0.0002$ ). 10  $\mu$ M histamine did not significantly affect extracellular nitric oxide metabolites ( $p = 0.2164$ ).

Intracellular nitric oxide metabolites were significantly increased by 0.25  $\mu$ M dexamethasone ( $p < 0.0001$ ), 10 mM L-citrulline ( $p = 0.0151$ ) and 10  $\mu$ M histamine ( $p = 0.04$ ).

Sample Name	Citrulline ( $\mu\text{M}$ )	Ornithine ( $\mu\text{M}$ )	Arginine ( $\mu\text{M}$ )
Control Cell Media	0	1.09 $\pm$ 0.60	196.18 $\pm$ 15.27
0.25 $\mu\text{M}$ Dex Cell Media	4.12 $\pm$ 1.79	6.8 $\pm$ 2.57	1242.28 $\pm$ 155.15
2 mM L-Arg Cell Media	0	12.99 $\pm$ 5.38	2758.55 $\pm$ 173.08
10 mM L-Cit Cell Media	5398.33 $\pm$ 738.95	2.04 $\pm$ 2.45	188.02 $\pm$ 8.59
10 $\mu\text{M}$ His Cell Media	0	0	252.43 $\pm$ 31.94

Table 5.5 Extracellular Amino Acid Concentrations in HUVEC Cells. Experiments were repeated in triplicate and are expressed as the average  $\pm$  the standard deviation.

Sample Name	Citrulline ( $\mu\text{M}$ )	Ornithine ( $\mu\text{M}$ )	Arginine ( $\mu\text{M}$ )
Control Cell Lysate	0	0	2.33 $\pm$ 1.86
0.25 $\mu\text{M}$ Dex Cell Lysate	0	0	0.56 $\pm$ 0.98
2 mM L-Arg Cell Lysate	0	0	53.37 $\pm$ 11.00
10 mM L-Cit Cell Lysate	241.78 $\pm$ 38.53	0	2.15 $\pm$ 1.87
10 $\mu\text{M}$ His Cell Lysate	0	0	2.34 $\pm$ 0.95

Table 5.6 Intracellular Amino Acid Concentrations in HUVEC Cells. Experiments were repeated in triplicate and are expressed as the average  $\pm$  the standard deviation.

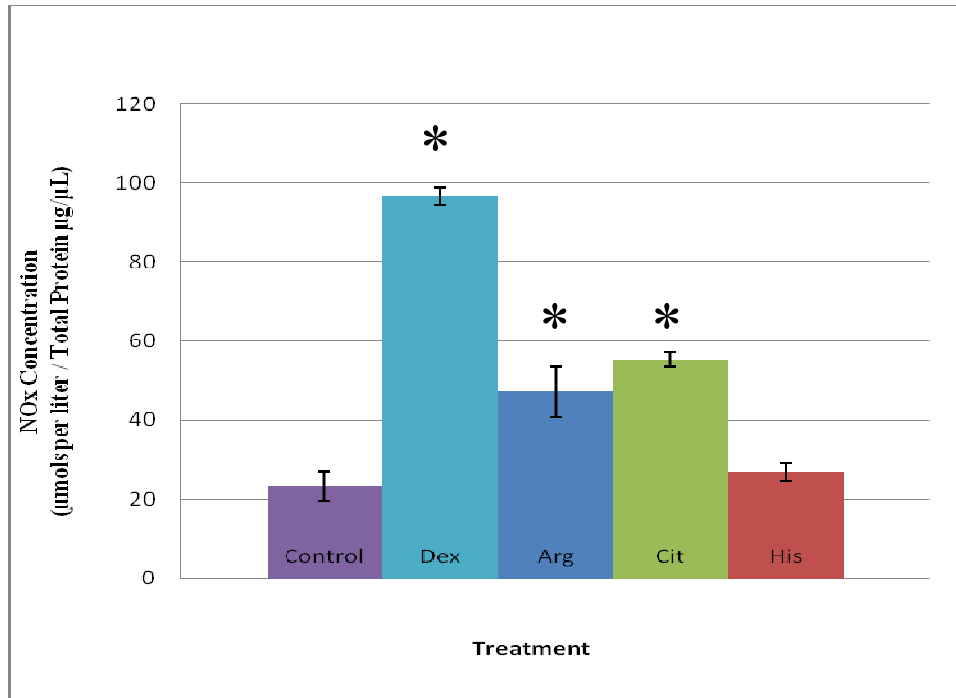


Figure 5.14 Changes in Extracellular Nitric Oxide Metabolites in HUVEC cells. Experiments were repeated in triplicate and are expressed as the average  $\pm$  the standard deviation. \* indicates p-value  $<0.05$ .

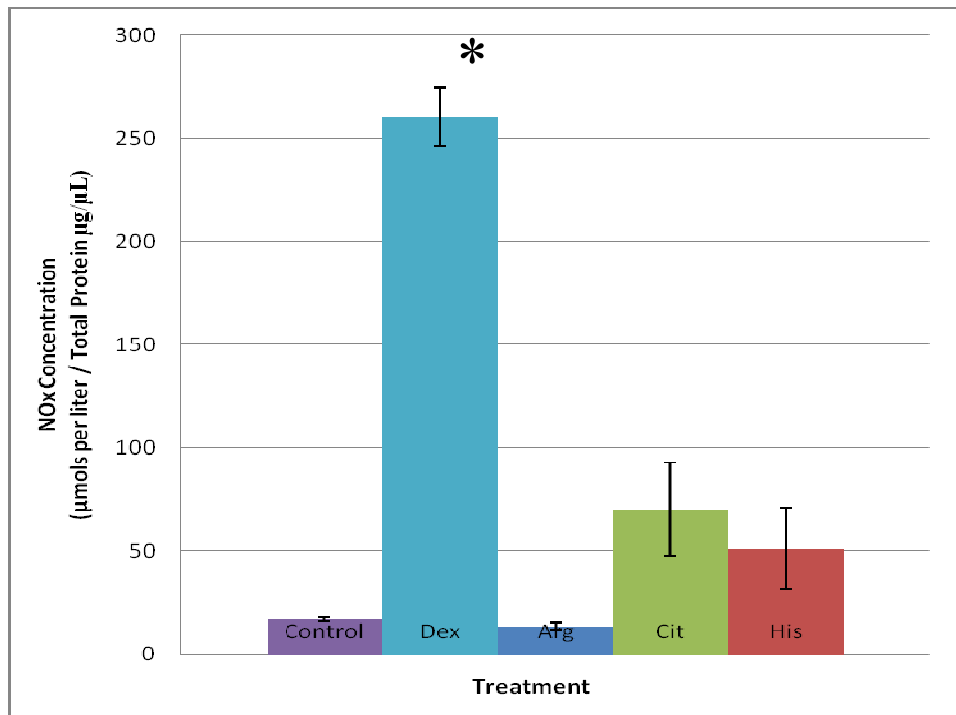


Figure 5.15 Changes in Intracellular Nitric Oxide Metabolites in HUVEC cells. Experiments were repeated in triplicate and are expressed as the average  $\pm$  the standard deviation. \* indicates p-value  $<0.05$ .

### *Summary of Results for HUVEC Cells*

HUVEC cells express mRNA for CPSI, NAGs, ASS, ASL, ARG2 and eNOS. It is interesting that 0.25  $\mu\text{M}$  dexamethasone increases citrulline concentrations from undetectable in the control sample to 4.12  $\mu\text{M}$  and increases arginine concentrations from 196.18  $\mu\text{M}$  in the control sample to 1242  $\mu\text{M}$ . This large effect might be due to an increase in enzyme activity. Intracellular amino acid concentrations did not vary significantly with stimuli. 0.25  $\mu\text{M}$  dexamethasone also had an impressive effect on both extracellular and intracellular nitric oxide metabolites, indicating its effectiveness at stimulating nitric oxide production. 10 mM L-citrulline and 10  $\mu\text{M}$  histamine were also significant in increasing nitric oxide metabolites intracellularly. Histamine is a known to increase nitric oxide production; our results confirm this result.



## CHAPTER VI

### INTERACTIONS OF ASS AND ASL ENZYMES

#### Introduction

The production of nitric oxide requires the precursor molecule arginine that is derived from the conversion of citrulline by argininosuccinate synthase and argininosuccinate lyase. There has been a growing body of data suggesting that free cytoplasmic arginine is not an efficient precursor of nitric oxide production in vivo studies (Baider, Aleshchenko, and Kuropteva, 2005; Bansal and Ochoa, 2003; Barr *et al.*, 2003; Barr *et al.*, 2007; Cederbaum, Yu, Grody, Kern, Yoo, and Iyer, 2004; Goodwin *et al.*, 2004; Hallemeesch *et al.*, 2002; Mori *et al.*, 2004; Romero, Platt, Caldwell, and Caldwell, 2006; Scaglia *et al.*, 2004; Summar *et al.*, 2004; Tentolouris *et al.*, 2004; Wu *et al.*, 1998). A model explaining nitric oxide production would account for this “arginine paradox” through the hypothesis that enzymes responsible for converting citrulline to arginine (ASS and ASL) form a protein complex with nitric oxide synthases (Figure 6.1) (Solomonson *et al.*, 2003). Enzyme complex formation and substrate channeling may play an important role in the molecular basis of vascular disorders. Proteins interact with each other in a highly specific manner, and protein interactions play a key role in many cellular processes; in particular the distortion of protein interfaces may lead to the development of many diseases (Ryan and Matthews, 2005). Protein interactions determine the outcome of many cellular processes. Therefore, the goal of this chapter is to identify and characterize protein-

protein interactions involved in the urea and nitric oxide cycles. We hypothesize that ASS and ASL interact with the nitric oxide synthases at the protein level.

## **Materials and Methods**

### *Cell Culture*

HEPG2 cells were grown in DMEM/F12 medium (Gibco) supplemented with 5% fetal bovine serum. HEK cells were grown in DMEM High Glucose Medium (Gibco) supplemented with 5% fetal bovine serum. HUVEC cells (Lonza) were grown in EGM Endothelial Growth Medium and supplemented with EGM SingleQuot Kit Supplements and Growth Factors (Clonetics). All cells were grown in an incubator at 37°C with 5% carbon dioxide (NuAIRE).

### *Confocal Imaging*

Confocal imaging was carried out in Dr. Judy Aschner's lab. Briefly, Cells were fixed and stained with the following ASS and eNOS primary antibodies and the appropriate secondary antibodies with a fluorescent tag obtained from Santa Cruz.

### *Co-Immunoprecipitation*

Total protein was isolated using Mammalian Protein Extraction Reagent (Pierce) using protease inhibitors PMSF (Sigma) and Protease Inhibitor Cocktail (Sigma) per manufacturer's instructions. Briefly, 10 µg of anti-ASS or anti-ASL antibody (Santa Cruz) was used to bait proteins which complex with ASS or ASL respectively. Antibodies were incubated with protein lysate for 4 hours with end-

over-end rotation at 4°C. 50% slurry of Sepharose G Beads (Sigma) was added and incubated with the protein lysate for 4 hours with end-over-end rotation at 4°C. Samples were centrifuged at 4°C and the lysate removed. The sample was washed three times with 1X TBS (Sigma).

#### *Western Blotting of Co-Immunoprecipitation Samples*

Protein was resuspended in 40 µL of Laemmli Blue (Bio-Rad) and 0.5% 2-beta mercaptoethanol (Sigma) and loaded onto a 4-20% Tris-HCl Ready Gel (Bio-Rad) and separated by electrophoresis at room temperature at 200 volts for 45 minutes. Protein was transferred to 0.45 micron nitrocellulose membranes (Bio-Rad) for 1 hour at 100 volts at 4°C. Membranes were blocked in 5% milk in TBS-T for 1 hour at room temperature and then probed with 1:1000 of appropriate primary antibody diluted in 5% milk in TBS-T overnight at 4°C. The membranes were washed 3 times with 0.5% TBS-T for 10 minutes and then incubated in 1:5000 dilution of secondary antibody diluted in 5% milk in TBS-T for 2 hours at room temperature. The membranes were washed 3 times with 0.5% TBS-T for 10 minutes. Proteins were visualized using ECL Western Blotting Substrate (Pierce). Each membrane was exposed to Kodak Bio-Max film (Perkin Elmer) and developed.

#### *Expression Constructs*

ASS cDNA was cloned into the TA vector using PCR techniques and the TOPO TA Cloning Kit (Invitrogen). The cDNA sequence was amplified such that a 5' Not1 site and 3' Sal1 site were incorporated into the cDNA sequence. The

sequence for the forward primer is: GAG GAG GCG GCC GCA TGT CCA GCA AAG GCT CCG TGG TTC TGG CCT (Invitrogen). The sequence for the reverse primer is: CGC CGC GTC GAC TTT GGC AGT GAC CTT GCT CG TGG TTC TGG CCT (Invitrogen). Positive clones were identified using restriction analysis, PCR analysis using internal primers for the ASS cDNA sequence and sequencing using M13 Forward (-20) and M13 Reverse Primers (Invitrogen). ASS cDNA sequencing forward primer: GCT TAT AAC CTG GGA TTG. ASS cDNA sequencing reverse primer: CCA GCT CCT CAT TGT ACA C. Positive clones were streaked on LB-agar plates containing 50 µg/mL kanamycin overnight at 32°C. Single colonies were chosen and grown in 2 mL LB-kanamycin (50 µg/mL) mini-cultures overnight at 32°C. Mini-cultures were added to 500 mL of LB-kanamycin (50 µg/mL) media and grown overnight at 32°C. DNA was isolated using PureLink™ Maxi Prep kit (Invitrogen). DNA was quantified by 260/280 reading on spectrophotometer. A restriction digest was performed to isolate the ASS cDNA using the internal restriction sites, NotI and SalI, and the DNA run on a 1% agarose gel for 4 hours at 40 volts. The ASS insert fragment was isolated using gel extraction, purified using the Wizard Kit (Promega) and resuspended in TE Buffer. The ASS insert fragment was dephosphorylated using CIAP and ligated to the pCTAP vector from the Interplay Mammalian TAP System (Stratagene) at a 2:1 ratio of insert:vector using 1 µg of linearized, dephosphorylated vector, 1 µL of T4 DNA ligase and 1 mM ATP (NEB) at room temperature for 10 minutes. Expression of constructs was verified by PCR analysis and western blot analysis.

### *Transfection of Mammalian Cells*

HEPG2 liver cells, HEK kidney cells and HUVEC endothelial cells were split and plated in 60 cm dishes at 70% confluency. The following day, 10 µg of purified DNA isolated from positively transformed bacteria using the Interplay Mammalian TAP System (Stratagene) was incubated with 500 µL of Opti-MEM Media (Invitrogen) and 40 µL of FugeneHD Transfection Reagent (Roche) for 15 minutes at room temperature before addition to plated cells. Cells were grown for 48 hours at 37°C with 5% CO<sub>2</sub>. Controls that were provided with the Interplay Mammalian TAP System kit were utilized. Mock transfections were performed using the following controls: no DNA and no transfection reagent.

### *Protein Isolation and Purification for Colloidal Coomassie Blue Staining*

Protein was isolated and purified using the Interplay Mammalian TAP System (Stratagene). Protein was resuspended in 40 µL of Laemmli Blue (Bio-Rad) and 0.5% 2-beta mercaptoethanol (Sigma) and loaded onto a 4-20% Tris-HCl Ready Gel (Bio-Rad) and separated by electrophoresis at room temperature at 200 volts for 45 minutes. Protein was transferred to 0.45 micron nitrocellulose membranes (Bio-Rad) for 1 hour at 100 volts at 4°C. Membranes were stained using the Colloidal Blue Staining (Invitrogen) for 3 hours at room temperature and then destained for at least 8 hours before imaging.

## Results and Discussion

### *Confocal Imaging*

ASS, ASL, and eNOS have previously been shown to co-localize to the caveolar compartment of vascular endothelial cells (Flam *et al.*, 2001).

Immunofluorescent confocal microscopy of piglet pulmonary endothelial cells supports previous data showing that the expression of ASS protein and eNOS protein are compartmentalized to the same location in the cell (Figure 6.1).

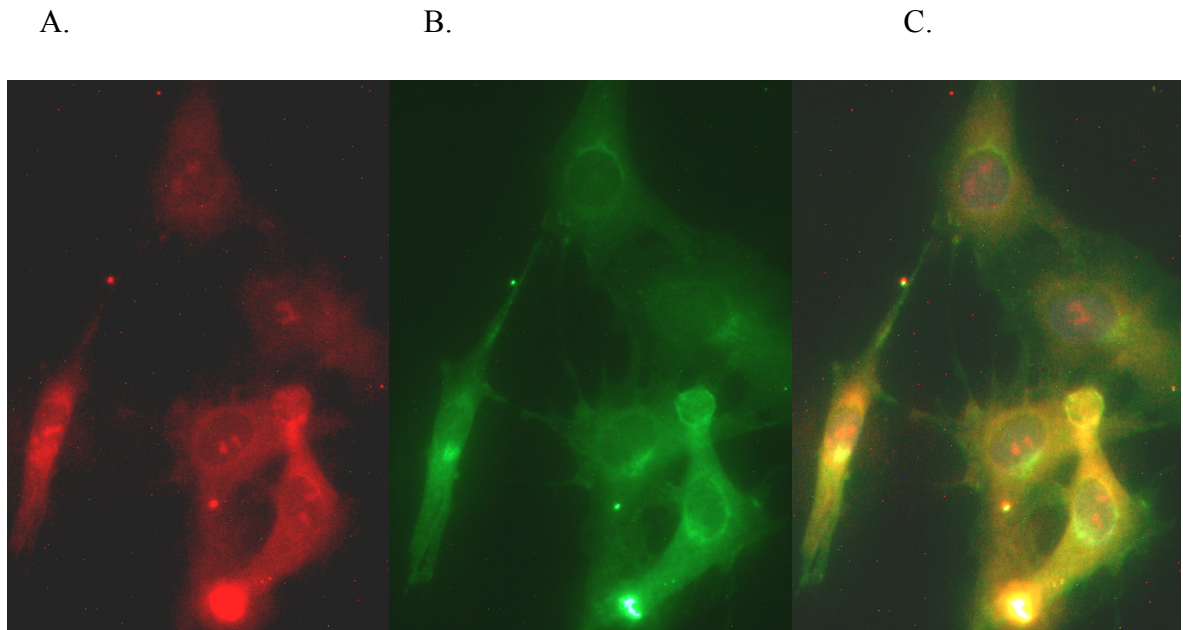


Figure 6.1 eNOS and ASS Overlap in the Cell. Panel A. piglet pulmonary endothelial cells stained with ASS. Panel B. piglet pulmonary endothelial cells stained with eNOS and Panel C. merged image. Confocal microscopy at 40X. Courtesy of Dr. Judy Aschner and Heng Zeng

## Co-Immunoprecipitation Experiments

### *Piglet Pulmonary Vascular Endothelial Cells*

Co-immunoprecipitation experiments in piglet pulmonary vascular endothelial cells reveal that eNOS and HSP90 co-immunoprecipitates with ASS. Furthermore when the co-immunoprecipitation experiment was repeated using ASL as the bait protein, eNOS and HSP90 co-immunoprecipitated suggesting that both ASL and ASS are able to interact with HSP90 and eNOS. This physical association suggests there may be channeling of substrate in this system. We propose that HSP90 acts as a scaffold, serving as a site where ASS, ASL and eNOS may dock to facilitate efficient substrate channeling to eNOS.

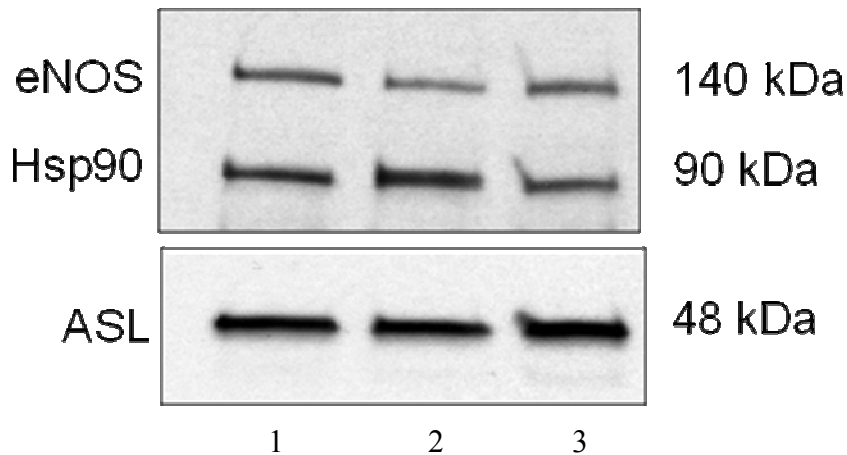


Figure 6.2 eNOS and HSP90 co-immunoprecipitate with ASL in piglet pulmonary endothelial cells. Co-immunoprecipitation experiment repeated in 3 independent experiments. Antibody against ASL was used to precipitate bound proteins and the immunoprecipitate was subject western blot analysis of ASL, HSP 90 and eNOS protein. Lanes 1-3 reflect the experiment repeated in triplicate, the same results observed in each experiment.

Figure courtesy of Dr. Judy Aschner and Heng Zeng.

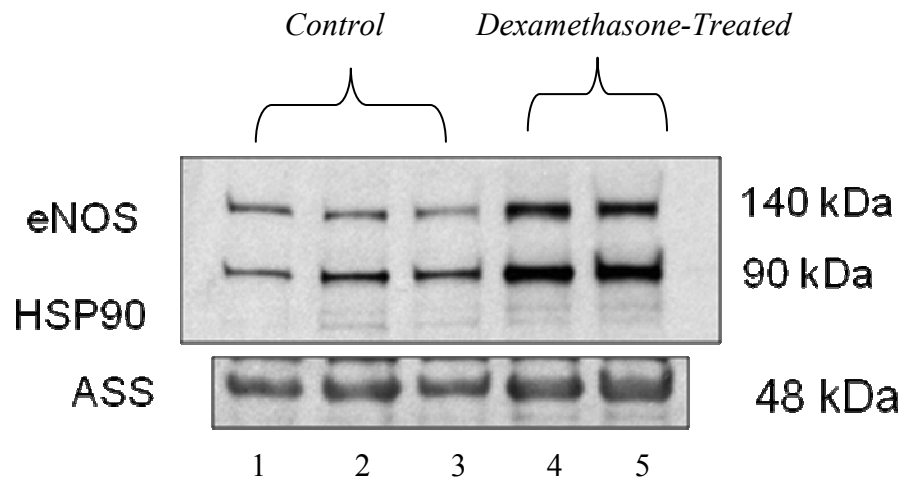


Figure 6.3 eNOS and HSP90 co-immunoprecipitate with ASS in piglet pulmonary endothelial cells. Antibody against ASS was used to precipitate bound proteins and the immunoprecipitate was subject western blot analysis of ASS, HSP 90 and eNOS protein. Lanes 1-3 reflect the experiment repeated in triplicate, the same results observed in each experiment. Lanes 4-5 reflect immunoprecipitation and western blot analysis in cells treated with 0.25  $\mu$ M dexamethasone for 24 hours.

Figure courtesy of Dr. Judy Aschner and Heng Zeng.



### *HEPG2 Liver Cells*

Co-immunoprecipitation experiments in HEK cells, HEPG2 cells and HUVEC cells did not reveal that ASS interacted with HSP90 or eNOS. These experimental results were negative (Figure 6.4). Lane 1 is normal cell lysate and both eNOS protein and HSP90 protein were detected in HEPG2 control cell lysate. Lane 2 is a co-immunoprecipitation using goat IgG as a control for background. Because the heavy and light chain of IgG falls within 25-75 kDa, the bands are visualized in the ARG1 and ASS western blot, but are not seen in the eNOS or HSP90 western blot because they are much larger proteins. Lanes 3 and 4 are co-immunoprecipitation experiments carried out using 10  $\mu$ g of ASS antibody. Bands were not detected in the eNOS or HSP90 western blot indicating that these proteins did not co-immunoprecipitate with ASS in this experiment. Furthermore, in the ASS western blot two bands are visualized close to 48 kDa, the smaller and more intense of the two bands is ASS protein and is indicated by an arrow. This same double banding pattern is not seen in the ARG1 western blot and therefore is specific to ASS.

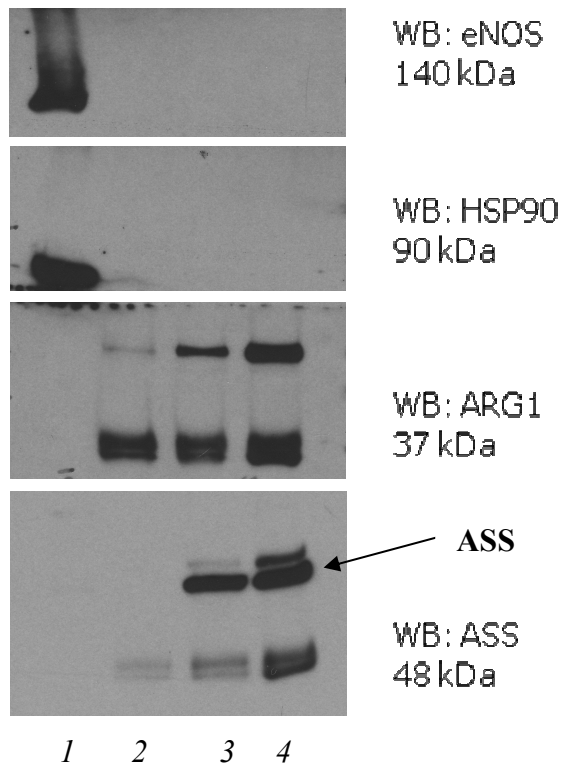


Figure 6.4 HSP90 and eNOS do not co-immunoprecipitate with ASS in HEPG2 cells. Antibody against ASS was used to precipitate bound proteins and the immunoprecipitate was subject western blot analysis of ASS, ARG1, HSP 90 and eNOS protein. Lane 1: Control Cell Lysate, Lane 2: 10  $\mu$ g anti-goat IgG; Lane 3: 10  $\mu$ g anti-ASS; Lane 4: 10  $\mu$ g anti-ASS

### *HEK Kidney Cells*

Co-immunoprecipitation experiments in HEK cells did not reveal that ASS interacted with HSP90 or eNOS. These experimental results were negative (Figure 6.5). Lane 1 is normal cell lysate and only HSP90 protein was detected in HEK control cell lysate. Lane 2 is a co-immunoprecipitation using goat IgG as a control for background. Because the heavy and light chain of IgG falls within 25-75 kDa, the bands are visualized in the ARG1 and ASS western blot, but are not seen in the eNOS or HSP90 western blot because they are much larger proteins. Lanes 3 and 4 are co-immunoprecipitation experiments carried out using 10  $\mu$ g of ASS antibody. Bands were not detected in the eNOS or HSP90 western blot indicating that these proteins did not co-immunoprecipitate with ASS in this experiment. Furthermore, in the ASS western blot two bands are visualized close to 48 kDa, the smaller and darker of the two bands is ASS protein and is indicated by an arrow. This same double banding pattern is not seen in the ARG1 western blot and therefore is specific to ASS. Additionally, a band was not visualized on the ARG1 western blot indicating that ARG1 does not interact with ASS in HEPG2 cells.

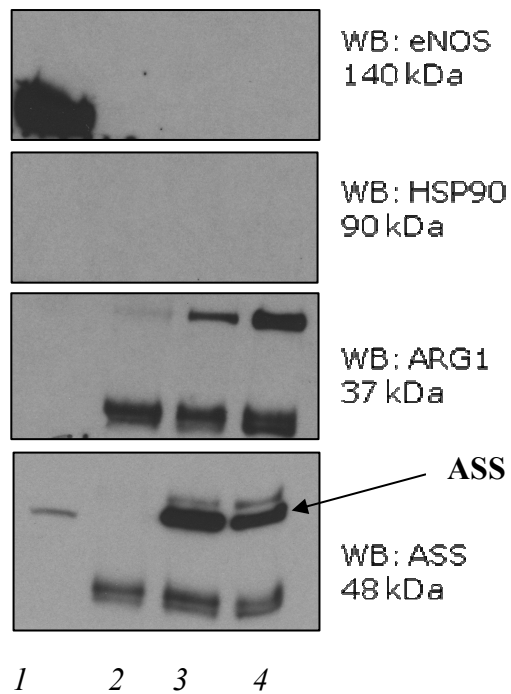


Figure 6.5 HSP90 and eNOS do not co-immunoprecipitate with ASS in HEK cells. Antibody against ASS was used to precipitate bound proteins and the immunoprecipitate was subject western blot analysis of ASS, ARG1, HSP 90 and eNOS protein. Lane 1: Control Cell Lysate, Lane 2: IgG; Lane 3: 10 µg ASS; Lane 4: 10 µg ASS

### *TAP Tag Coomassie Results*

A second method used to determine protein interactions involving ASS, was tandem affinity purification (TAP) tagging. We designed a construct in which we tagged the ASS protein with strepavidin and calmodulin (Stratagene Mammalian Tap Tag Kit), transfected the construct into HEPG2 and HEK cells and purified the ASS bait protein and interacting protein partners. We ran the protein on an SDS gel and stained the gel with coomassie to visual the proteins. As a control, cells were transfected with Mef protein per the manufacturer's instructions (Stratagene).

### *Verification of Expression of ASS Tap Tagged Protein in HEPG2 and HEK Cells*

HEPG2 and HEK cells were transfected with the ASS-Tap Tagged protein using FugeneHD per manufacturer's instructions (Roche). Cell lysates were harvested 24 and 48 hours post-transfection and protein was analyzed using western blot analysis against the ASS protein. Tagged ASS was detected 24 and 48 hours in both cell lines. HEPG2 and HEK cells were also transfected with MEF protein, which was also detected 24 and 48 hours post-transfection in HEPG2 and HEK cells (Figure 6.6).



Figure 6.6 Validation of ASS-Tap Tagged Construct. Cells were transfected with 10  $\mu$ g of ASS-Tap Tagged DNA or Mef-Tap Tagged Control DNA and grown for 24 or 48 hours. Western blot analysis of ASS and Mef proteins revealed that both HEPG2 and HEK cells expressed a measurable amount of transcript 48 hours post-transfection.

### *HEPG2 Liver Cells*

A coomassie stain of the interacting proteins indicated that there are potential proteins that interact with ASS in HEPG2 cells compared with the HEPG2 control cell lysate (Figure 6.7 Lane 3 compared with Lane 2). We conducted western blot analysis probing with antibodies for eNOS, HSP90 and ASL (Figure 6.8). We detected both eNOS and HSP90 in the HEPG2 control cell lysate (Lane 1), but were unable to detect these proteins in our experimental ASS-tap tagged sample (Lane 2). We detected a faint band in the lane for ASL (indicated with an arrow) and we detected two bands for ASS, one band for the normal size protein and one for the tagged protein. This experiment supports the co-immunoprecipitation experiments that HSP90 and eNOS do not interact with ASS in HEPG2 cells. However, ASL may interact with ASS in HEPG2 cells.

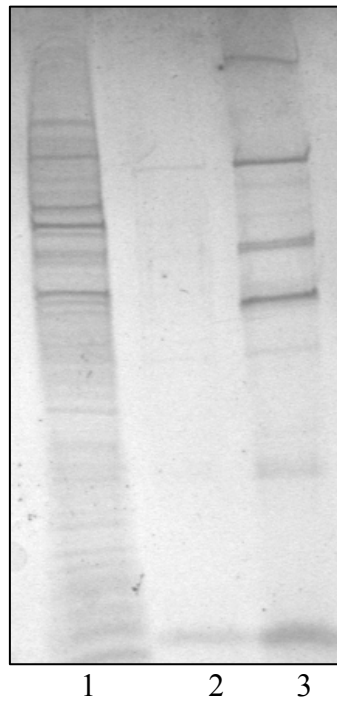


Figure 6.7 Coomassie Stain of ASS Tap Tag Results in HEPG2 Cells. Cells were transfected with 10  $\mu\text{g}$  of ASS-Tap Tagged DNA or Mef-Tap Tagged Control DNA and grown for 48 hours. The cell lysate was purified and interacting proteins isolated and run on an SDS-Page gel which was stained with Colloidal Coomassie (Invitrogen). Lane 1: Protein Plus Ladder (Invitrogen), Lane 2: MEF Tagged Lysate; Lane 3: ASS-Tagged Lysate



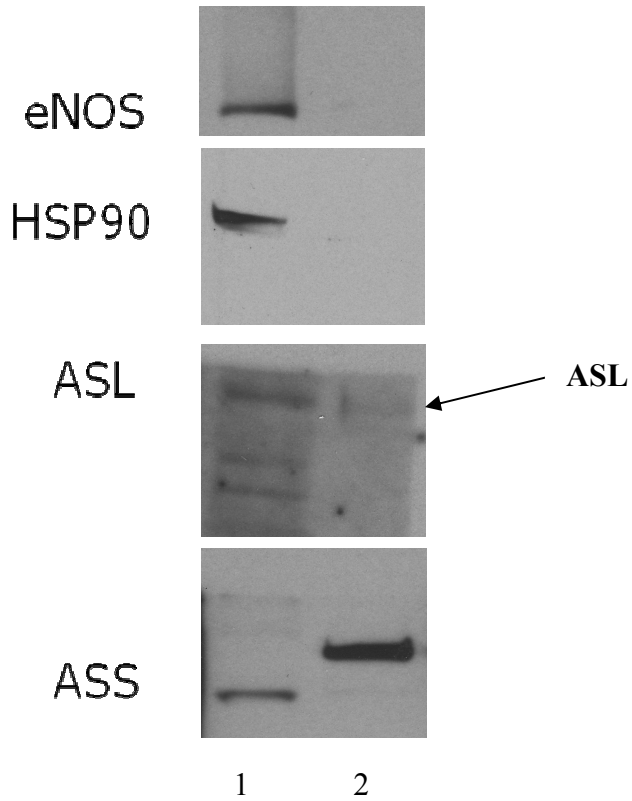
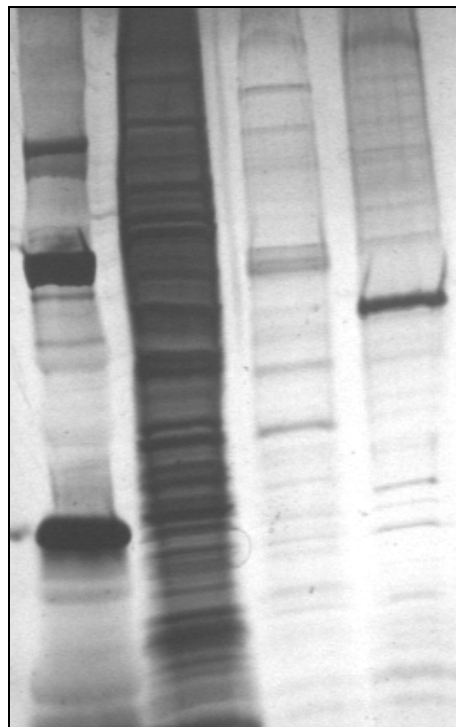


Figure 6.8 Western Blot Analyses of Protein Interactions in ASS-Tag Transfected Cells. Cells were transfected with 10  $\mu\text{g}$  of ASS-Tag Tagged DNA grown for 48 hours. The cell lysate was purified and interacting proteins isolated and western blot analysis was used to identify ASS, ASL, HSP90 and eNOS in the control and transfected cells. Lane 1: HEPG2 Control cell Lysate; Lane 2: ASS-Tagged Lysate

### *HEK Kidney Cells*

A coomassie stain of the interacting proteins indicated that there were proteins that interact with ASS in HEK cells compared with the HEK control cell lysate (Figure 6.9). HEK cells did not express eNOS protein by western blot analysis. Western blot analysis of the transfected cells indicated that neither HSP90 nor ASL interact with ASS in HEK cells. HSP90 and ASL were detected in the control lysate but not in the ASS tagged transfected samples (Figure 6.10).



1 2 3 4

Figure 6.9 Coomassie Stain of ASS Tap Tag Results in HEK Cells. Cells were transfected with 10  $\mu$ g of ASS-Tap Tagged DNA or Mef-Tap Tagged Control DNA and grown for 48 hours. The cell lysate was purified and interacting proteins isolated and run on an SDS-Page gel which was stained with Colloidal Coomassie (Invitrogen). Lane 1: Protein Plus Ladder (Invitrogen), Lane 2: Control HEK Cell Lysate, Lane 3: Mef-tagged Lysate; Lane 4: ASS-Tagged Lysate

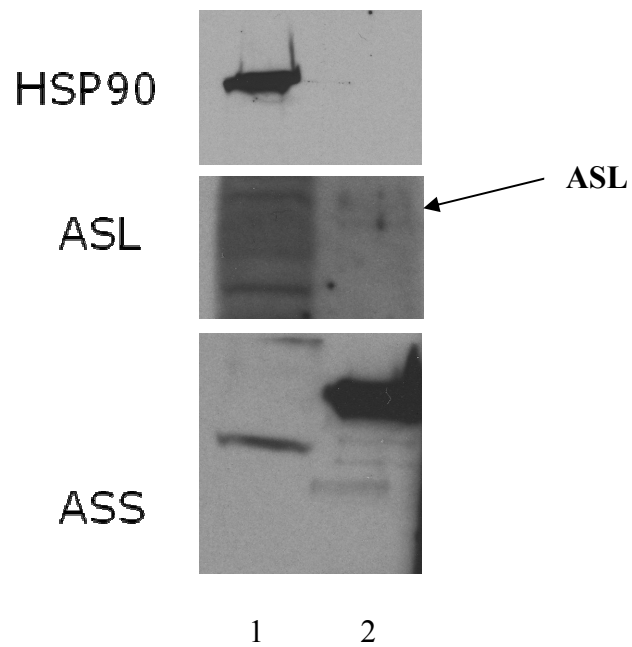


Figure 6.10 Western Blot Analyses of Protein Interactions in ASS-Tag Transfected Cells. Cells were transfected with 10  $\mu$ g of ASS-Tag Tagged DNA grown for 48 hours. The cell lysate was purified and interacting proteins isolated and western blot analysis was used to identify ASS, ASL and HSP90 in the control and transfected cells. Lane 1: HEK Control cell Lysate; Lane 2: ASS-Tagged Lysate

### *HUVEC Endothelial Cells*

HUVEC cells are primary umbilical vein cells and are notoriously difficult to transfect through traditional transfection methods such as FugeneHD. Our coomassie experiments indicated that the cells did not take up the ASS tagged construct through transfection. Future experiments aimed at transfecting primary cells would be beneficial.

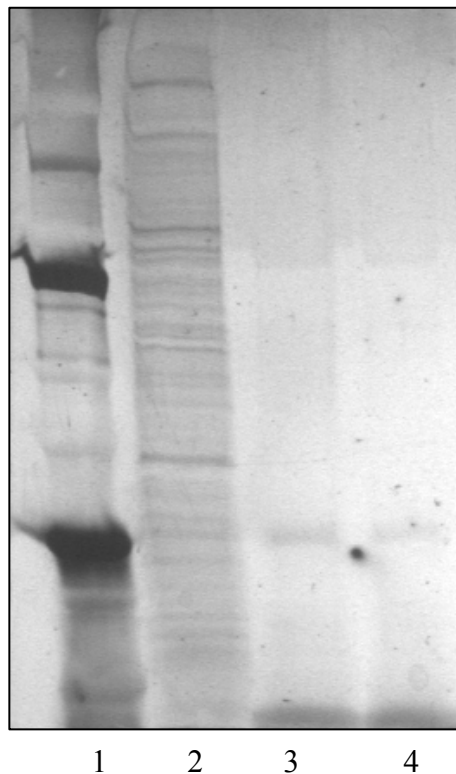


Figure 6.11 Coomassie Stain of ASS Tap Tag Results in HUVEC Cells. Cells were transfected with 10  $\mu\text{g}$  of ASS-Tap Tagged DNA or Mef-Tap Tagged Control DNA and grown for 48 hours. The cell lysate was purified and interacting proteins isolated and run on an SDS-Page gel which was stained with Colloidal Coomassie (Invitrogen). Lane 1: Protein Plus Ladder (Invitrogen), Lane 2: Control HUVEC Cell Lysate, Lane 3: Mef-tagged Lysate; Lane 4: ASS-Tagged Lysate

## Summary

This specific aim was conducted under the hypothesis that ASS and ASL interact with other proteins *in vitro*. Specifically, our interest is in the enzymes that utilize the substrates created by ASS and ASL. Confocal imaging studies revealed that ASS and eNOS co-localize within piglet primary pulmonary endothelial cells. This evidence supports work conducted by other researchers who are also intrigued by this pathway (Flam *et al.*, 2001). We also believe that ASL is co-localized to this area, but further study is needed to prove that hypothesis. We have shown that in piglet primary pulmonary vascular endothelial cells ASS and ASL independently co-immunoprecipitate with HSP90 and eNOS, leading to the hypothesis that ASS and ASL may interact with each other directly in this cell type. Furthermore, our data show that dexamethasone treatment may increase nitric oxide production as seen in Chapter 6, through an increase in complex formation. An increase in HSP90 and eNOS precipitated protein was detected in a sample of piglet pulmonary endothelial cells using ASS as the bait protein. Results from HEPG and HEK cell lines indicate that this complex may not form in these cell types. However, TAP tagging revealed proteins that were purified using the ASS-tagged sample. Future studies revealing the identity of these proteins need to be conducted. However western blot analysis of ASS-TAP tagged HEPG2 and HEK cells revealed a band for ASL, indicating that these two proteins may interact in both the HEK and HEPG2 cells. It is possible that the ASS-eNOS-HSP90 interaction only occurs in primary cell types or that the interaction is weak – which might explain the lack of detectable interactions in the HEPG2 and HEK cells. The results from this Chapter support our hypothesis that the

cytoplasmic enzymes of the urea and nitric oxide cycles form a metabolic complex. Additional evidence in support of this complex in other cell types needs to be addressed in future work.

## CHAPTER VII

### SUMMARY AND FUTURE DIRECTIONS

Diverse cellular processes are mediated by dynamic network of interacting proteins in living organisms. These proteins carry out important metabolic functions in the cell. The urea and nitric oxide cycles are only one, out of many interactions between metabolic pathways. Understanding the regulation of these (and other) metabolic enzymes at the transcriptional, protein and protein-protein interaction levels will provide important clues to help elucidate the function of a known or novel proteins and further elucidate the relationships between metabolic pathways. The term “metabolon” was coined by a scientist named Srere in 1985 to denote associating complexes of sequential metabolic enzymes (Srere, 1987) and many metabolons have been identified both in humans and plants (Jorgensen, Rasmussen, Morant, Nielsen, Bjarnholt, Zagrobelny, Bak, and Moller, 2005; Velot *et al.*, 1997; Watford, 1989). It is thought that through this the formation of metabolic complexes, cells are able to increase their metabolic efficiency and manage cellular processes to save space and energy. The interactions between enzymes give rise to a higher level of complexity that must be accounted for when one wishes to understand the regulation of metabolism and diseases associated with metabolic enzymes.

Urea cycle disorders have typically been considered the result of single gene mutations (Engel *et al.*, 2009; Gao *et al.*, 2003; Jackson *et al.*, 1986; Mitchell *et al.*, 2009; Summar *et al.*, 2008) and until recently, the impact of a deficiency of these

enzymes on other metabolic pathways hasn't been considered. Clinically, patients with a urea cycle deficiency have lower circulating levels of urea cycle intermediates (arginine and citrulline) (Pearson *et al.*, 2001) and lower levels of nitric oxide production (Barr *et al.*, 2003; Scaglia *et al.*, 2004), suggesting a relationship at the molecular level between these two pathways.

We formulated a hypothesis that the cytoplasmic enzymes involved in the urea cycle exist as a metabolon with the nitric oxide synthase enzymes. In this metabolon, citrulline is the preferred substrate for nitric oxide production and it enters the cell on solute neutral carrier 1 and is then sequentially acted upon by ASS, AS and a nitric oxide synthase nitric oxide, regenerating citrulline in the process. There is increasing evidence that disruption of protein interactions play a significant role in disease. Therefore, because the enzymes of the urea cycle have the potential to affect many disease conditions, these pathways were examined for evidence of protein interactions.

Through our studies in the mouse model, we determined that ASS and ASL mRNA transcripts are expressed at similar levels in the heart, lung, spleen, kidney and liver. mRNA transcript levels of ASS and ASL did not correlate with protein levels, which may be a result of complex transcript or protein regulation. Measurements of tissue citrulline and nitric oxide metabolites showed a positive correlation between citrulline concentration and nitric oxide metabolite concentrations. Conversely, we did not detect a correlation between tissue arginine and tissue nitric oxide metabolites, suggesting that citrulline may be a preferred substrate for endogenous nitric oxide production. These findings support the clinical



observation that citrulline is an effective substrate for improving endogenous nitric oxide production in patients (Ananthakrishnan *et al.*, 2009; Barr *et al.*, 2007; Smith *et al.*, 2006; Summar, 2001).

Urea cycle enzymes have traditionally been thought of as tissue specific enzymes localized to the liver, where the urea cycle occurs. Our second aim was to determine which human tissues expressed transcripts for the urea and nitric oxide cycle enzymes. It was hypothesized that these enzymes would be localized to tissues in which they were metabolically active. Using quantitative real-time PCR (qRT-PCR) and RNA from normal human tissues, metabolic roles were assigned to the tissues based upon which enzymes were expressed in which tissues. In liver cells, the full complement of urea cycle enzymes was detected – which indicates that liver cells are capable of utilizing the full urea cycle. All of the enzyme transcripts were expressed in the kidney except for ARG1 and iNOS. This would permit the cycle to process nitrogen to the point of generating arginine. Without the enzyme ARG1, arginine is free to exist in the cell, to exit the cell or to become a building block for protein catabolism. In the heart tissue, transcripts for all of the enzymes were detected except for iNOS and nNOS. Given the mRNA expression of the complete set of urea cycle enzymes, the heart would have the potential to carry out a complete urea cycle, if the proteins for these enzymes were expressed. The detection of high transcript expression for ASS, ASL and eNOS, given expression at the protein level, would permit the heart to sustain endogenous nitric oxide production. This is important for the heart to permit sustained vascular flow. In this chapter, we discovered that the urea cycle enzymes are expressed in many more tissues than

previously thought. It will be important to further investigate the unknown role and/or function of these enzymes in different tissue types.

Using the differential transcript expression in human tissues, we chose to examine the effects of known regulators of the urea and nitric oxide cycles on the *in vitro* expression of ASS and ASL mRNA transcript expression, protein levels, and levels of the urea and nitric oxide cycle intermediates (arginine and citrulline) using a cell culture model. Discordant mRNA transcript and protein expression for the enzymes studied suggest that the regulation of these enzymes is complex and likely occurs at the transcriptional through post-translational levels. The lack of correlation between mRNA transcript and protein expression is becoming an increasingly reported phenomenon that scientists are still trying to understand (Guo, Xiao, Lei, Deng, Xiao, Liu, Chen, Li, Wu, Chen, Jiang, Tan, Xie, Zhu, Liang, and Deng, 2008; Gygi, Rochon, Franza, and Aebersold, 1999; Honoki, Dohi, Tabata, Mii, Miyauchi, Tsutsumi, Tsujiuchi, Morishita, Miura, Moriyama, and ., 1993; Maier, Guell, and Serrano, 2009; Su, Pan, Zhou, Harvey, Hunger, and Kilberg, 2008). The relationship between gene expression measured at the mRNA level and the corresponding protein level is itself an important piece of data that is necessary for the understanding of molecular regulation of metabolic pathways. Currently, these data are being compiled into databases in hopes of designing machine learning tools able to predict protein expression from mRNA transcript data and vice versa (Greenbaum *et al.*, 2003; Raghava and Han, 2005). Furthermore, the treatment of cells with citrulline increased nitric oxide in each of the cell types studied which supports both clinical and laboratory studies showing that citrulline administration is

able to ameliorate nitric oxide production (Ananthakrishnan *et al.*, 2009; Barr *et al.*, 2003; Pearson *et al.*, 2001; Smith *et al.*, 2006; Smith *et al.*, 2005; Summar, 2001; Summar *et al.*, 2004). This increase in nitric oxide metabolites was not detected with L-arginine treatment, suggesting that citrulline may be the preferred precursor for nitric oxide production, thus supporting the metabolon hypothesis. It is our hypothesis that arginine is not able to enter the complex, but is an intermediate generated from citrulline through the sequential actions of ASS and ASL and utilized directly by a nitric oxide synthase to produce nitric oxide. Our experiments yielded some interesting, unexpected and previously unreported results. For example, our results show that histamine can induce inducible nitric oxide transcripts expression in both HEK and HEPG2 cells. This may be a potential mechanism for the induction of inducible nitric oxide in the immune response. Furthermore, histamine increased transcript expression of all of the urea cycle enzymes in the HEPG2 liver cells suggesting that the urea cycle may be affected by changes in immune function. Finally, treating the cells with dexamethasone increased the production of nitric oxide in each of the cell lines studied without a corresponding increase in mRNA or protein expression. Combined with the increase in nitric oxide production seen in citrulline-treated cells, we suggest that a possible mechanism by which dexamethasone increases nitric oxide production is through a potential increase in metabolon formation. This would permit citrulline to be used for continued nitric oxide production.

Our final aim was to demonstrate that ASS and ASL interact with each other and/or other proteins to form multi-protein complexes and that these multi-protein

complexes differ in their composition by cell type. Confocal microscopy results show that ASS and eNOS localize to the same area of the cell. Co-immunoprecipitation experiments revealed an interaction between ASS, HSP90 and eNOS and a separate interaction between ASL, HSP90 and eNOS in piglet primary vascular endothelial cells. Furthermore, co-immunoprecipitation experiment which revealed increased protein expression of pulled-down complex proteins eNOS and HSP90 with ASS in dexamethasone treated piglet pulmonary endothelial cells supporting our hypothesis that these enzymes form a complex and that dexamethasone may increase nitric oxide production by increasing the aggregation of these enzymes. Although the interaction between ASS, HSP90 and eNOS and ASL, HSP90 and eNOS were not detected in HEPG2 or HEK cell, tandem affinity purification experiments revealed that undetermined proteins do interact with ASS in both of these cell lines. A future aim of this work is to identify these interacting protein partners.

Investigators are just beginning to address protein-protein interactions in nitrogen metabolism. Some have pointed to the importance of the formation of a complex of enzymes (Flam *et al.*, 2001;Solomonson *et al.*, 2003). It is plausible that protein interactions are involved in the complex regulation of urea and nitric oxide cycle enzymes and that these interactions may affect the activity of these enzymes. The experiments completed in this work support the hypothesis that the cytoplasmic enzymes of the urea and nitric oxide cycles form a complex which produces nitric oxide. This work is only the beginning in an important era of translational research addressing the complexities of metabolic pathways and their effects on clinical

disease. Future directions need to determine the precise proteins that are interacting with each of the urea and nitric oxide cycle enzymes. Furthermore, this insight should be followed with an analysis of the precise regions of each enzyme that are necessary and/or sufficient for interactions with other proteins. Finally, because these enzymes have different biological functions in different tissues it does not suffice to examine these interactions in only one tissue type. It will be important to classify and categorize the interactions of these enzymes and proteins in many tissue and cell types. The overlap between metabolic pathways and disease presents a complexity that will require the combined efforts of new technology, molecular biology, clinical diagnostic skills and machine learning tools to parse out the intricate details. Metabolic pathways hold the key to understanding the molecular underpinnings of many diseases, once we further understand these pathways we can apply our knowledge to create better medical prevention and treatment.

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