THE PHYSIOLOGICAL IMPACT OF ORAL ARGININE SUPPLEMENTS IN MALE SPRAGUE DAWLEY AND ZUCKER DIABETIC FATTY RATS

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ABSTRACT

Oral arginine supplements have become popular in recent years due to their proposed vasodilatory effect *via* the nitric oxide synthase pathway. L-arginine, however, is also a substrate for three other enzymes – arginase, arginine decarboxylase, and arginine:glycine amidinotransferase producing urea, agmatine, and creatine, respectively. Interestingly, both L- and D-arginine have also been found to be effective *in vitro* scavengers of the toxic glucose and fructose metabolite methylglyoxal. Yet, despite the widespread use of oral arginine supplements, the impact of L- and D-arginine on arginine metabolic pathways and basal methylglyoxal production remains unclear. We thus sought to investigate the effect of L-arginine and D-arginine, each at 500 mg/kg/day in drinking water administered for 4 weeks and 1000 mg/kg/day for 16 weeks to separate groups of 9-week-old male Sprague Dawley (SD) rats. As arginine supplements have been shown to be more effective in a disease state, we also supplemented Zucker lean and Zucker Diabetic Fatty (ZDF) rats with 1000 mg/kg/day of oral L- or D-arginine for 12 weeks. We then quantified the expression of arginine related enzymes and their metabolites in the plasma, urine and various organs/tissues. Additionally, we assessed the effect on basal methylglyoxal levels and its degradation by the glyoxalase pathway, relevant cardiovascular parameters, and oral glucose tolerance. L-arginine significantly decreased cationic amino transporter 1 expression in the ileum of both SD and ZDF rats. L-arginine also significantly increased eNOS expression in the aorta and kidney of SD and ZDF rats but only decreased mean arterial pressure in ZDF rats. Arginase expression was decreased in the ileum of SD rats by L-arginine but increased in ZDF rats. Interestingly, L-arginine decreased arginase activity in the ileum of ZDF rats. Plasma creatinine levels were unaffected by either L-or D-arginine in the plasma of SD rats. Oddly, D-arginine, which is thought to be inert, was able to alter the expression/activity of enzymes and metabolites of all four arginine metabolic pathways. L-arginine generally maintained or decreased methylglyoxal (MG) levels in both SD and ZDF rats. In ZDF, but not SD rats, L-arginine decreased levels of methylglyoxal in the plasma. Oppositely, D-arginine generally increased MG levels except for in the ileum and lungs of SD rats. Finally, neither isomer proved beneficial in ameliorating impaired glucose tolerance of ZDF rats. In conclusion, both L- and D-arginine significantly affected enzymes and metabolites in several pathways that use arginine as a substrate. As a vasodilator, L-arginine appears to be more effective in a pathological rather than physiological model. Finally, the efficacy of L- and D-arginine as *in vivo* scavengers of MG will require further investigation.

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DEDICATION

To my beautiful sisters

Lisa Koffman and Rachel Koffman

"If you know you are on the right track, if you have this inner knowledge, then nobody can turn you off... no matter what they say." – Barbara McClintock, winner of the 1983 Nobel Prize in Physiology or Medicine

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LIST OF ABBREVIATIONS

ADC	Arginine decarboxylase

ADMA	Asymmetric dimethyl arginine
AGAT	Arginine: glycine amidinotransferase
AGE	Advanced glycation end products
AMPK	AMP-activated protein kinase
ASL	Arginosuccinate lyase
ASS	Arginosuccinate synthase
ATP	Adenosine triphosphate
BP	Blood pressure
BSA	Bovine serum albumin
CAT-1	Cationic amino transporter 1
CEL	N^{ε}-carboxyethyl-lysine
cGMP	Cyclic guanosine monophosphate
CO	Carbon Monoxide
Cr	Creatine
D-ARG	D-arginine
EAA	Nutritionally essential amino acids
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
FAA	Functional amino acids
GAMT	Guanidinoacetate *N*-methyltransferase
GLUT	Glucose transporter
HUVEC	Human umbilical vascular endothelial cell
iNOS	Inducible nitric oxide synthase
IL	Interleukins
IV	Intravenous
L-ARG	L-arginine
L-ORN	L-ornithine
MAP	Mean arterial pressure
MG	Methylglyoxal
MODIC	Methylglyoxal-derived imidazolium cross-link

MOLD	Methylglyoxal-lysine dimers
NEAA	Nutritionally non-essential amino acids
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
nNOS	Neuronal nitric oxide synthase
NOS	Nitric oxide synthase
NO_x	Nitrate + nitrite
ODC	Ornithine decarboxylase
RAEC	Rat aortic endothelial cells
RAGE	Receptor for advanced glycation end products
ROS	Reactive species
SD	Sprague Dawley
SEAA	Semi-essential amino acids
SHR	Spontaneously hypertensive rats
T2DM	Type 2 diabetes mellitus
TBST	Tris buffered saline with tween
TMB	3,3', 5,5' Tetramethylbenzidine
UV	Ultraviolet
VSMC	Vascular smooth muscle cells
WAT	White adipose tissue
WKY	Wistar Kyoto
ZDF	Zucker diabetic fatty
ZLC	Zucker lean controls
ZOA	Zucker obese + L-arginine
ZOC	Zucker obese controls
ZOD	Zucker obese + D-arginine

CHAPTER 1. INTRODUCTION

1.1 Amino Acids

Amino acids are the building blocks of proteins. These organic molecules consist of three functional groups surrounding a central or alpha carbon. Two groups, the basic amino group and the acidic carboxyl group are common to all amino acids. Oppositely, the organic R group is a side chain which is exclusive to each amino acid (Reddy, 2018).

Traditionally, there are 20 standard amino acids. Amino acids can be classified in various ways but are most commonly grouped based on the polarity of their R group (Reddy, 2018). Thus, amino acids are listed as either polar, non-polar, acidic or basic. Proteins are formed by linking amino acids *via* peptide bonds which form through a condensation reaction. When this occurs the amino and carboxyl groups are no longer functional (Reddy, 2018). Thus, the overall ionization and function of a protein is determined by the R groups of the amino acids of which it is comprised (Bhagavan, 2002).

All amino acids, glycine excluded, exist as enantiomers. Though L and D configurations exist, proteins are almost entirely composed of L-amino acids (Bhagavan, 2002). This is due to the evolution of protein synthesis enzymes to exclusively use L-enantiomers (Reddy, 2018). However, at the microscopic levels, bacteria are known to use D-amino acids for a variety of functions such as synthesizing proteins required in their cell wall (Cava et al., 2011).

Beyond protein synthesis amino acids are precursors for a variety of nitrogenous molecules. For example, amino acids are required to produce purine and pyrimidine nucleotides which are the building blocks for nucleic acids, precursors of nucleotide cofactors and important energy carriers (Moffatt & Ashihara, 2002). Furthermore, amino acids are not just building blocks of molecules. They can serve as neurotransmitters, such as glutamate which is the most common excitatory neurotransmitter in mammals (Meldrum, 2000). Their derivatives can be used as hormones such as thyroxine which is produced from tyrosine in the thyroid gland (Crockford, 2009). Finally, several amino acids such as arginine function as intermediates in processes like the urea cycle (Ash, 2004).

1.1.1 Essential, semi-essential and non-essential amino acids

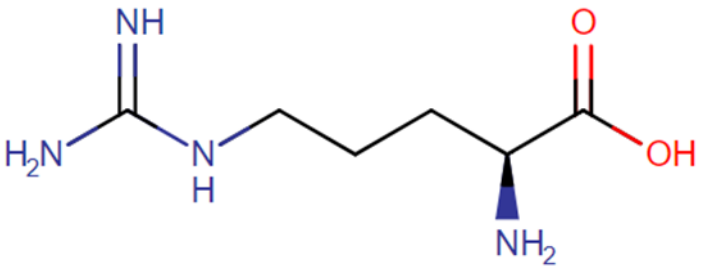
From a nutritional standpoint amino acids have been traditionally divided into two groups: nutritionally essential amino acids (EAA) and nutritionally non-essential amino acids (NEAA)

(Guoyao Wu, 2009). EAA are those whose carbon skeletons cannot be synthesized in the body or cannot be synthesized *de novo* at a high enough rate to meet the body's needs. Thus, EAA must be consumed in the diet to ensure adequate amounts are available (Guoyao Wu, 2009). NEAA are amino acids which can be produced *via de novo* synthesis at rates sufficient to support proper body functioning and thus do not need to be ingested in the diet (Hou et al., 2015).

While these two categories have been long accepted, there is no evidence that NEAA are synthesized in the body at a rate adequate to fully support growth, reproduction and optimal health (Hou et al., 2015). Instead, studies have provided evidence that many NEAA cannot be synthesized fast enough to maintain the body's metabolic needs under both physiological and pathological conditions (Hou et al., 2015). For example, studies on arginine have shown that this traditionally considered NEAA may in fact be essential. One such study on male reproduction, showed that men fed an arginine-deficient diet for nine days had a decrease in both sperm motility and number (L. J. Holt & Albanese, 1944). This study not only showed that arginine is required for optimal spermatogenesis, but that functional uses of amino acids beyond their role in nitrogen balance need to be considered when determining essentiality of an amino acid (Hou et al., 2015). This study and many like it (Meléndez-Hevia et al., 2009; Sevastiadou et al., 2011; Tanimura, 1967; Zeng et al., 2008) have provided evidence for a new category of amino acids known as conditionally or semi essential amino acids (SEAA). SEAA acids are involved in many different areas of physiology ranging from growth, to cell signalling as well as metabolism. They are not considered essential because they can be synthesized *de novo,* however, they are not non-essential because the primary determinant of their plasma levels is dietary intake (Appleton, 2002).

1.2 L-Arginine

Figure 1.1. Chemical structure of L-arginine.



(Image source: https://www.drugbank.ca/structures/DB00125/image.svg)

1.2.1 Overview

L-Arginine (L-Arg) is a basic (Reddy, 2018), semi-essential (Appleton, 2002), functional (Guoyao Wu, 2010) amino acid (Fig. 1.1). Arginine is not considered an EAA because it can be produced *de novo via* precursors such as glutamine, glutamate and proline (Appleton, 2002). However, it is not NEAA because its plasma levels are primarily based on dietary intake (Castillo et al., 1994). Furthermore, under stressful arginine depleting conditions such as inflammation (van der Zwan et al., 2011) and infection (Senft, 1967) endogenous arginine biosynthesis rates do not increase and dietary arginine is required. Endogenous arginine is primarily produced *via* the intestinal-renal axis (Dhanakoti et al., 1990). Essentially, epithelial cells of the small intestine produce citrulline which is released into the blood circulation and taken up by the proximal cells of the kidney which then convert citrulline into arginine (Dhanakoti et al., 1990). Additionally, small amounts of arginine are produced in the liver *via* the urea cycle (Sidney & Morris, 1992). Thus, arginine depletion can also result under conditions of kidney dysfunction (Y.S. et al., 2015), intestinal dysfunction (Crenn & Cynober, 2010) or liver damage (Sidney M Morris, 2012).

In healthy adults, arginine levels typically range from ~80-120 μmol/L in the plasma (Sidney M Morris, 2007) and 100-1000 μmol/g in tissues (Castillo et al., 1996). A dietary intake of 1-3 g of L-Arg per day is recommended (Closs et al., 2004), however, studies have found ~4.4 g/day to be the mean dietary intake of adult males and females (King et al., 2008; Mirmiran et al., 2016). Good sources of dietary arginine include nuts and legumes, with grains containing lower concentrations of this semi-essential amino acid (Vega-López et al., 2010). However, in 2016, Mirmiran *et al*. reported that L-Arg dietary intake by human subjects was primarily sourced from grains followed by meats, dairy and lastly nuts and legumes (Mirmiran et al., 2016).

1.2.2 Pharmacokinetics: Absorption and Distribution

Dietary arginine is absorbed *via* the splanchnic region (Castillo, Chapman, Yu, et al., 1993). The intestinal brush border uses the y+ transporter system for cationic amino acids to actively uptake arginine *via* cationic amino transporters (CAT) such as CAT-1 (White, 1985). However, due to high arginase activity in the small intestine, 40% of enteral arginine is degraded *via* first-pass metabolism prior to reaching systemic circulation (Guoyao Wu et al., 2007). As arginine is a substrate for many enzymes it is quickly degraded in the bloodstream. One study found that differences in arginine serum concentrations could not be detected when blood samples were

collected more than 4 hours after oral or intravenous (i.v.) arginine supplementation. This suggests that exogenous arginine is not stored in the blood and furthermore maintaining elevated serum concentrations requires dosing every 4 hours (Guoyao Wu et al., 2007). In agreement with this finding, a much older study estimated arginine to have a plasma half-life of ~1 hour (Noeh et al., 1996).

The bioavailability of arginine has not been highly reported and varies between studies. For example, one study investigated the pharmacokinetics of oral and i.v. arginine supplementation in healthy human subjects and reported an average bioavailability of 21±4% (Tangphao et al., 1999). Yet another study which also used healthy human subjects reported a much higher bioavailability of 68±9 (51–87)% (Bode-Böger et al., 1998). Finally, at the midpoint of these values Castillo *et al* reported an oral bioavailability of ~38% in adult humans (Castillo, Chapman, Yu, et al., 1993).

Like arginine bioavailability, research on arginine distribution is limited. In one study rats received 0.8g/kg intraperitoneal injections of arginine. Arginine concentrations within the five tissues assayed peaked between 15 and 30 min post injection (Noeh et al., 1996). Highest concentrations were found in the pancreas followed by the vena cava, bronchi, heart and finally the aorta. Peak plasma arginine concentrations were observed 15 min post arginine injection. By 120 min post arginine injection, plasma and most tissues had arginine concentrations similar to those observed in the saline injected control group (Noeh et al., 1996). Another study investigated the distribution of oral and i.v. arginine supplementation in the liver, brain and testes (Campistron et al., 1982). Significant increases in all three tissues were observed following both oral and i.v. arginine administration. Interestingly, the oral route provided a greater increase in arginine concentrations in both the brain and testes as compared to i.v. administration. Within the liver up to 15% of the total arginine dose was detected, with a majority of it protein bound (Campistron et al., 1982). Interestingly, the liver itself does not appear to play a large role in absorbing supplemental arginine as hepatocytes virtually lack the y+ transporter system. For this reason, only 10% of arginine delivered to the liver is taken into the organ (G Wu & Morris Jr., 1998).

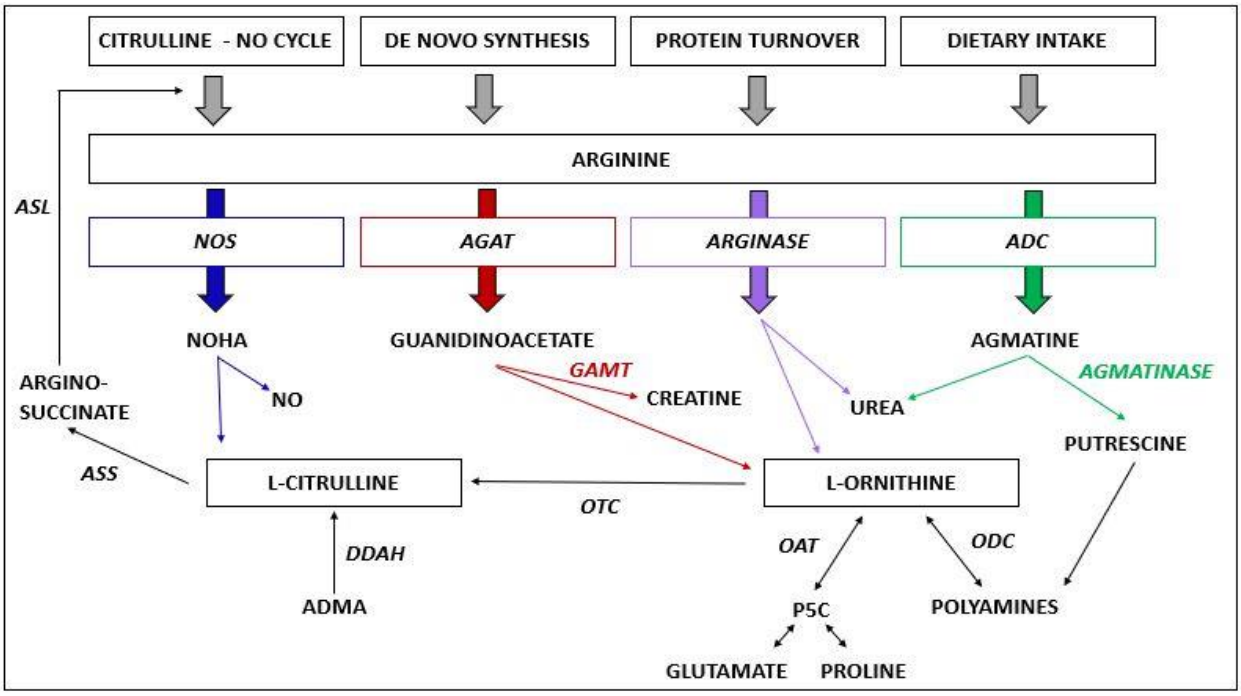


Figure 1.2. Arginine sources and metabolic pathways.

Arginine pools can be fed *via de novo* synthesis, dietary intake, protein turnover and recycling of L-citrulline *via* the citrulline – NO cycle. Four main metabolic pathways convert arginine into a wide variety of products. Abbreviations: ADC – arginine decarboxylase; ADMA – asymmetric dimethylarginine; AGAT – arginine: glycine amidinotransferase; ASL – arginosuccinate lyase; ASS – arginosuccinate synthase; DDHA – dimethylarginine dimethylaminohydrolase; GAMT – guanidinoacetate N-methyltransferase; NO – nitric oxide; NOHA – N^{ω}-hydroxy-l-arginine; NOS – nitric oxide synthase; OAT – ornithine amino transferase; ODC – ornithine decarboxylase; OTC – ornithine transcarbamylase; P5C - L-Δ^1-pyrroline-5-carboxylate.

1.2.3 Metabolism: Enzymatic Pathways

L-Arg is a substrate for a variety of enzymes making it one of the most multifunctional amino acids in the body. While its metabolism is complex (Fig. 1.2), four main pathways utilize arginine to create a wide range of metabolic products. These pathways include the nitric oxide synthase (NOS), arginase, arginine decarboxylase (ADC) and arginine:glycine amidinotransferase (AGAT) pathways (Sidney M. Morris, 2006). *In vivo* these pathways interact in many ways. For example, endothelial nitric oxide synthase (eNOS) and arginase compete for L-Arg as a substrate.

Additionally, intermediary products of the NOS pathway act to inhibit arginase (G Wu & Morris Jr., 1998). Furthermore, some pathways, for example, the ADC and arginase pathways produce common products such as polyamines (Fig.2, Sidney M Morris, 2007). While it is simplest to analyze each pathway individually, these interactions must also be considered when making conclusions based on *in vivo* data.

1.2.4 Nitric Oxide Synthase Pathway

Endothelial nitric oxide synthase (eNOS) is one of three NOS isoforms (Förstermann & Sessa, 2012) which catalyzes the conversion of L-Arg to nitric oxide (NO) and L-citrulline (Fig.2, Palmer et al., 1988). This constitutively expressed enzyme resides mainly in the endothelial cells of arteries. Expression is highest in larger arteries as compared to smaller arteries with no expression observed in capillary endothelial cells (Heiss et al., 2015). Additionally, eNOS is expressed in a majority of blood cell types including platelets, leukocytes, erythrocytes and endothelial progenitor cells (Heiss et al., 2015).

The NO produce by NOS enzymes has a very short half-life and can only travel short distances before being oxidized. Thus, NO is considered an autacoid as it must act close to the site of production (Lundberg, 2006). NO produced by eNOS within vascular endothelial cells maintains many aspects of vascular homeostasis such as endothelium-dependent vasodilation, vascular repair, angiogenesis and prevention of platelet activation and adhesion (Lundberg, 2006). For example, NO synthesized within endothelial cells diffuses to nearby vascular smooth muscle cells (VSMC) where it increases cyclic guanosine monophosphate (cGMP) *via* G-protein-mediated signalling. This increase in cGMP leads to vascular smooth muscle cell relaxation and ultimately vasodilation (Archer et al., 1994).

L-Citrulline produced by eNOS can be recycled into L-Arg *via* the citrulline-NO cycle (Fig. 2, Sidney M Morris, 2007). Essentially, L-citrulline produced by eNOS is catalyzed by argininosuccinate synthase (ASS) to argininosuccinate. Following this rate-limiting step, argininosuccinate lyase (ASL) converts argininosuccinate into L-Arg which is then utilized by eNOS to complete the cycle (Flam et al., 2007). Both ASS and ASL have been extensively studied in hepatocytes as they participate in the urea cycle (Shambaugh 3rd. G.E., 1977). However, with the discovery of the citrulline-NO cycle, greater interest has been taken in regards to these enzymes' function within endothelial cells. It has been found that these enzymes co-localize with

eNOS in the caveolae of endothelial cells (Flam et al., 2001). Furthermore, there is evidence that this cycle is closely and essentially coupled with endothelial NO production (Flam et al., 2007).

NO can react to form a variety of products such as peroxynitrite, nitrous anhydride and its final metabolites nitrite and nitrate (Habib & Ali, 2011). Quantifying NO levels is difficult due to the molecule's very short half-life. Thus, nitrite and nitrate levels are often quantified together as a means of measuring NO production (Giustarini et al., 2008). Finally, NO production can be hindered by the endogenous eNOS inhibitor asymmetric dimethylarginine (ADMA), a product of methylated nuclear protein proteolysis. By competing with L-Arg, ADMA can result in decreased NO production *via* reduced eNOS activity (Leone et al., 1992).

1.2.5 Arginase Pathway

Arginase exists in two isoforms, arginase I and arginase II (Jenkinson et al., 1996; Li et al., 2002). In humans these isozymes are related 58% by sequence identity (Morris et al., 1997). Arginase I is expressed almost exclusively within the cytosol of the liver where it aids in ammonia detoxification *via* the urea cycle (Sidney M Morris, 2007). However, arginase I can also be found in lesser amounts in endothelial and vascular smooth muscle cells (Sidney M Morris, 2007). Oppositely, arginase II is a mitochondrial enzyme found in non-hepatic tissue with expression being highest in the kidneys, pancreas and small intestine (Choi et al., 2012). Additionally, arginase II can be found in vascular endothelial cells (Bachetti et al., 2004). While its function is relatively unknown, arginase II most likely serves to regulate polyamine, proline and NO synthesis (Morris, 2005).

Both arginase I and II catalyze the breakdown of L-Arg to L-ornithine (L-ORN) and urea (Fig. 2, Ash, 2004). Urea is the final product of ammonia detoxification (Shambaugh 3rd. G.E., 1977). L-ORN is a precursor for polyamines and proline which promote cell proliferation and collagen synthesis, respectively (S M Morris, 2004). While ornithine decarboxylase (ODC) is considered the rate limiting step for polyamine synthesis, a variety of studies have shown arginase to be a regulating factor in this process (H Li et al., 2001; Hui Li et al., 2002; Wei, et al, 2001).

As polyamines are involved in cell proliferation they must be tightly regulated to ensure proper growth and development without promoting tumorigenesis. Arginase has been shown to be intimately involved in this physiological balance. For example, during pregnancy the activity of myometrial arginases has been found to increase ~25-fold in order to supply sufficient levels of

polyamines for the developing fetus (Weiner et al., 1996). However, overactive arginase activity has also been linked to cancer. Studies have now shown that inhibiting arginase in different cancer cell lines results in a suppression of cancer cell proliferation (Shatanawi & Qasrawi, 2017; Singh et al., 2000). In an attempt to control this, L-ORN and urea exert negative feedback inhibition on arginase (Hunter A, Downs, 1945).

Additionally, researchers have taken interest in potential pathologies that may arise from increased proline synthesis due to enhanced arginase activity or expression. L-ORN produced by arginase can only be converted to proline in cells that express ornithine aminotransferase (Morris, 2007). Such cells include vascular smooth muscle cells. When arginase activity is increased in these cells there is a resultant increase in proline and proline-rich proteins such as collagen. This increased collagen synthesis can lead to pathological levels of collagen deposition, ultimately causing vascular lesions (Durante et al., 2007). Furthermore, collagen plays an integral role in other processes including wound healing (Albina et al., 1988; Campbell et al., 2013), tissue remodelling (Durante, 2013; Pera et al., 2014) and fibrosis (Pera et al., 2014; Wynn, 2004).

1.2.6 Arginase-NOS Interaction:

The arginase and NOS enzymes exhibit a complex interplay that remains to be fully understood. Both enzymes utilize L-Arg as a substrate creating a competition between the two (Morris, 2004). NOS enzymes have an affinity ~1000 times greater than arginases. However, due to arginases having a V_{max} ~1000 times greater than that of NOS enzymes, arginases can still effectively compete for L-Arg and decrease NO production (Guoyao Wu & Morris, 1998). Many studies have exhibited this substrate competition and linked it to endothelial dysfunction (Johnson, 2004; Rodriguez et al., 2000; Romero et al., 2008; Shin et al., 2012; Zhang et al., 2004). Additionally, the ability of arginases to outcompete NOS enzymes does not appear to be affected by the compartmentalization of L-Arg (Elms et al., 2013). Oppositely, NOS is able to competitively inhibit arginase *via* the NO intermediate N^{ω}-hydroxy-l-arginine (Boucher et al., 1994; Daghigh et al., 1994). The ability of N^{ω}-hydroxy-l-arginine to inhibit arginase has been demonstrated *in vitro* in both macrophages (Chenais et al., 1993) and endothelial cells (Buga et al., 1996). Finally, NO produced by NOS enzymes has the ability to inhibit ODC, a downstream enzyme of the arginase pathway, and thus can affect polyamine production (Bauer et al., 2001).

1.2.7 Arginine Decarboxylase Pathway

ADC metabolizes L-Arg into agmatine which can be further broken down by agmatinase to produce the polyamine putrescine and urea (Fig. 2, Xiaoqiu Wang et al., 2014). Putrescine can then be ultimately converted to the polyamines spermine and spermidine. Traditionally, ADC was thought to only be expressed in non-mammalian tissues and cells such as plants, bacteria and invertebrates (Zhu et al., 2004). Within the last 30 years ADC has been discovered in various mammalian tissues (Lortie et al., 1996; Sastre et al., 1998; Wang et al., 2014) including the rat brain (Li et al., 1995) and liver (Horyn et al., 2005). In the past decade existence of a putative ADC has been reported in humans with expression being highest in the brain (Zhu et al., 2004).

Similarly, agmatinase was not originally thought to be a mammalian enzyme. Today, studies have provided evidence of mammalian agmatinase (Li et al., 1995; Sastre et al., 1996) and human agmatinase has been cloned (Iyer et al., 2002; Mistry et al., 2002). The highest levels of agmatinase mRNA in humans are in the liver and kidney (Mistry et al., 2002). However, it is important to note that agmatine is also a substrate for oxidases (Ascenzi et al., 2002; Cabella et al., 2001; A. Holt & Baker, 1995). The proportion of agmatine that is metabolised by agmatinase has not been thoroughly investigated in mammalian cells. One study has demonstrated that despite rat hepatocytes having the highest levels of agmatinase mRNA, oxidases are the primary route of agmatine catabolism in these cells (Cabella et al., 2001). Thus, it is possible that agmatinase is not the major route for agmatine catabolism in mammals.

The ADC-agmatinase pathway has been found to serve as an alternative pathway for polyamine production (Xiaoqiu Wang et al., 2014). This was demonstrated in a study in which ODC_1 was knocked out in ovine conceptus trophectoderm leaving the ADC-agmatinase pathway as the only source of polyamines for the developing fetus. The authors found that one half of ODC_1 knock out conceptuses were developmentally abnormal. In the half that developed normally, increases in ADC and agmatinase mRNA were observed along with increased translation of agmatinase mRNA. Thus demonstrating that while the ODC pathway is the major route for polyamine synthesis, the ADC-agmatinase pathway can be upregulated when compensation is required (Xiaoqiu Wang et al., 2014).

Agmatine has been proposed to have a variety of physiological roles which have been sorted into three main categories: acting as a neurotransmitter (Reis & Regunathan, 2000), inhibiting cell proliferation (Ishizuka et al., 2000; Satriano et al., 1999) and inhibiting NOS enzymes (Galea et

al., 1996; Satriano et al., 2001). In brief, studies have found agmatine to be a ligand for α_2 adrenergic (Li et al., 1994; Molderings et al., 2000), imidazole (Li et al., 1994; Molderings et al., 1997; Piletz et al., 1995), N-methyl-D-aspartate (Askalany et al., 2005; Yang & Reis, 1999), and nicotinic acetylcholine receptors (Loring, 1990). Agmatine inhibits cell proliferation by inducing the production of antizyme, a protein which inhibits ODC as well as polyamine transporters. Agmatine is the only molecule other than polyamines that is known to induce antizyme (Satriano et al., 1998). Finally, agmatine has been shown to inhibit neuronal NOS (nNOS) and inducible NOS (iNOS), but stimulate eNOS (Piletz et al., 2013). Inhibition of nNOS occurs through increased hydrogen peroxide production *via* agmatine mediated NAPDH oxidation which irreversibly inactivates the enzyme (Demady et al., 2001), iNOS inhibition occurs *via* an aldehyde metabolite of agmatine (Satriano et al., 2001) and eNOS stimulation has been demonstrated but the mechanism of action remains unclear (Morrissey & Klahr, 1997; Mun et al., 2010).

1.2.8 Arginine:Glycine Amidinotransferase Pathway

AGAT catalyzes the conversion of L-Arg to guanidinoacetate and L-ORN, the former of which is then metabolized into creatine (Cr) *via* guanidinoacetate *N*-methyltransferase (Fig. 2, GAMT, Brosnan & Brosnan, 2004)). Transport of Cr between cells occurs through the Cr transporter 1 and is coded by the gene SLC6A8 (Yu et al., 2013). AGAT is a mitochondrial enzyme which (Magri et al., 1975) exists as a dimer in rats (McGuire et al. 1980) and humans (Gross et al., 1986). In adult mammals, AGAT is primarily expressed in the kidney and pancreas with highest activity being observed in the kidney, pancreas and brain (Van Pilsum et al., 1972). Highest expression of GAMT occurs within the liver and pancreas (Olivier Braissant et al., 2005) with highest activity in the liver, pancreas and kidney (Van Pilsum, Stephens, Taylor, et al., 1972). AGAT but not GAMT mRNA expression and activity is downregulated when serum Cr levels are increased either through endogenous synthesis or dietary supplementation. Additionally, L-ORN is a potent inhibitor of AGAT (Wyss & Kaddurah-Daouk, 2000).

Due to the distribution of these two enzymes, Brosnan & Brosnan (2004) proposed that guanidinoacetate produced in the kidney by AGAT is released into the bloodstream where it is then converted in the liver to Cr by GAMT. However, in humans the liver may be able to produce Cr all on its own (Brosnan & Brosnan, 2004) as one paper found human hepatocytes to express high levels of AGAT mRNA (X.-R. Xu et al., 2001). Within the pancreas both AGAT and GAMT are

expressed. When investigating pancreatic Cr synthesis, Da Silva *et al*. (2014) found high AGAT activity and thus guanidinoacetate production. However, in comparison the rate of Cr synthesis was relatively low in the pancreas due to low GAMT activity. It was therefore, speculated that the pancreas serves as a source of guanidinoacetate which is delivered to the liver for Cr synthesis *via* the hepatic portal vein (Da Silva et al., 2014). Finally, high activity of both AGAT and GAMT has also been observed in the brain. As astrocytes, which compose the blood brain barrier, lack SLC6A8, Cr synthesis within the brain is believed to occur endogenously (O. Braissant & Henry, 2008).

Approximately, 95% of Cr in the human body is found within skeletal muscle with the remaining ~5% in the brain and testes (Kreider et al., 2017). Cr mainly functions as a transient energy source by combining with a phosphoryl group (P_i) to form phosphorylated Cr (PCr) *via* creatine kinase (CK). When adenosine triphosphate (ATP) needs to be resynthesized, hydrolysis of PCr to Cr and a phosphoryl group can provide the energy required to phosphorylate adenosine diphosphate back into ATP (Kreider et al., 2017). By maintaining adequate levels of ATP Cr plays an important in role in anaerobic exercise (Kreider et al., 2017). Additionally, the CK/PCr energy shuttle enhances energy metabolism by linking ATPases which utilize ATP to sources of ATP synthesis such as glycolysis and mitochondrial oxidative phosphorylation (Kreider et al., 2017). In the brain creatine functions to buffer and transport high energy-phosphates as well as aid in cone growth migration and neurotransmitter release. Additionally, creatine is required for electrophysiological functions such as Na^+/K^+-ATPase activity, maintaining membrane potential, Ca^{2+} homeostasis and restoring ion gradients (Wyss & Kaddurah-Daouk, 2000).

In vertebrates, creatinine is produced from the non-enzymatic cyclization of Cr. *In vitro* the equilibrium between Cr and creatinine depends on both pH and temperature. For example, Cr is favoured at low temperatures and a basic pH (Wyss & Kaddurah-Daouk, 2000). *In vivo* Cr and PCr are converted at a rate of ~1.7% /day and excreted in the urine. Cr appears to be the only source of creatinine. Additionally, creatinine is a poor substrate of the Cr transporter and is membrane permeable allowing it to constantly diffuse out of tissues into the bloodstream where it is eventually excreted by the kidneys (Wyss & Kaddurah-Daouk, 2000). Finally, the rate of non-enzymatic conversion of Cr to creatinine is constant, thus increases in Cr levels from increased endogenous production or ingestion of meat in the diet would result in directly proportional increases in creatinine (Wyss & Kaddurah-Daouk, 2000).

1.2.9 Excretion

Elimination of both oral and i.v. L-Arg supplements from the plasma occurs in a biphasic manner. Substantial urinary excretion of L-Arg supplements appears to only occur at very high concentrations of the amino acid, once the renal threshold for reabsorption has been an exceeded (Tangphao et al., 1999). For example, Tangphao *et al*. (1999) only observed substantial urinary excretion in humans following i.v. infusion with 30 g of L-Arg but not 10 g of oral L-Arg. Furthermore, urinary excretion only occurred within the first 90 min at a rate of 1.17 ml min^{-1} kg^{-1} following infusion after which a much slower, presumably non-renal elimination of plasma L-Arg occurred. Similarly, pigs have shown enhanced urinary excretion of L-Arg following supplementation due to a renal reabsorption maximum at the distal loop of Henle (Dantzler & Silbernagl, 1993).

According to Brosnan and Brosnan (2004), at an average arginine plasma concentration of 90 μmol/L and a glomerular filtration rate of 190 L/d the human kidneys filter and reabsorb ~ 3.1 g of arginine a day. Transport of arginine out of the renal tubule across the apical border occurs *via* the $b^{0,+}$ system. An antiport transporter reabsorbs arginine in exchange for neutral amino acids. This transporter is also responsible for the reabsorption of other cationic amino acids such as lysine and ornithine in addition to cysteine (Wagner et al., 2001). Arginine is then transported across the basolateral membrane into peritubular capillaries *via* the y^+L system which also carries lysine and ornithine but not cysteine (Brosnan & Brosnan, 2004). Like most other amino acids this reabsorption is thought to occur in the proximal tubule (Silbernagl & Deetjen, 1972).

1.3 Functional Roles of Arginine In The Body

1.3.1 Functional Amino Acids

Functional amino acids are a relatively new class of amino acids which are defined by Wu (2010) as *"those that participate in and regulate key metabolic pathways to improve health, survival, growth, development, lactation and reproduction of the organism"*. Essentially, these amino acids play important roles beyond solely maintaining nitrogen balance and participating in metabolic pathways. Functional amino acids can be EAA, NEAA or SEAA (Guoyao Wu, 2013). In mammals arginine is considered a functional amino acid due to its extreme versatility as a substrate and regulatory molecule (Hou et al., 2015).

1.3.2 Regulation of Gene Expression

Arginine supplementation has been shown to exert multiple effects on the body. Some of these effects are made possible by arginine's ability to regulate gene expression (W. Jobgen, Fu, et al., 2009; W. Jobgen, Meininger, et al., 2009). Such regulation in mammals can occur through a variety of mechanisms. For example, amino acids are known to activate transcription factors and CCAAT/enhancer-binding protein. They also inhibit gene expression by targeting specific regulatory sequences such as nutrient sensing-response element and amino acid response element (Brasse-Lagnel et al., 2009).

Studies on diet induced obesity in male Sprague Dawley (SD) rats showed that dietary L-Arg supplementation reduced mRNA levels for fatty acid binding protein 1, glycogenin, protein phosphatase 1B, caspases 1 and 2, and hepatic lipase, but increased expression of peroxisome proliferator-activated receptor gamma, heme oxygenase 3, glutathione synthetase, insulin-like growth factor II, sphingosine-1-phosphate receptor, AMP-activated protein kinase (AMPK) and stress-induced protein in white adipose tissue (W. Jobgen, Fu, et al., 2009). Many of these alterations in expression have positive impacts in regards to reducing obesity and oxidative stress.

For example, heme oxygenase 3 oxidizes heme into biliverdin and carbon monoxide, the latter of which activates guanylyl cyclase to produce cGMP (W. Jobgen, Fu, et al., 2009). The cGMP signalling pathway then enhances lipolysis and acetyl-CoA oxidation in adipose tissue (W. S. Jobgen et al., 2006). Caspases are involved in inflammation and apoptosis (Fernández & Lamkanfi, 2015), and by reducing expression of caspase 1 and 2 oxidative stress may be reduced. Additionally, enhanced expression of glutathione synthetase has been shown to increase production of the antioxidant glutathione which may further ameliorate oxidative stress (Lu, 2013). In a similar study Zucker diabetic fatty (ZDF) rats supplemented with L-Arg showed increased expression of genes involved in fatty acid oxidation such as AMP-activated protein kinase, nitric oxide synthase 1 (NOS-1, nNOS) and proliferator-activated receptor gamma coactivator-1α (, Fu et al., 2005).

Arginine also has the ability to regulate genes which code for enzymes and proteins involved in its own metabolism (S M Morris, 2004). For example, excess arginine suppresses the expression of ASL and ASS (Schimke, 1964), while low extracellular arginine is known to inhibit iNOS expression *via* decreased translation of mRNA (Lee et al., 2003). Low arginine levels also result in the upregulation of CAT-1 *via* increased transcription of the CAT-1 gene (SLC7A1, Fernandez

et al., 2003; Xia et al., 2016) and enhance initiation of CAT-1 mRNA translation (J Fernandez et al., 2001).

1.3.3 Involvement in Cell Signalling Pathways

Functional amino acids such as arginine can modulate cellular signaling pathways involving mammalian target of the rapamycin (Yao et al., 2008), AMPK, extracellular kinase, Jun kinase, mitogen-activate protein kinase and gaseous molecules including NO, carbon monoxide (CO) and hydrogen sulfide (Li et al., 2009). For example, in porcine skeletal muscle, dietary arginine supplementation increased the phosphorylation of 4E-binding protein-1 (4E-BP1) which when phosphorylated dissociates from the inactive eukaryotic initiation factor-4E-binding protein-1 complex. Released eukaryotic translation initiation factor 4E can then bind with eukaryotic translation initiation factor 4G to form the active eukaryotic translation initiation factor 4G - eukaryotic translation initiation factor 4E complex leading to enhanced protein synthesis and whole-body growth *via* the 43S ribosomal complex (Yao et al., 2008).

Arginine is also intimately involved in gaseous signalling. As the substrate for NO, L-Arg has been shown to increase mammalian NO synthesis in a variety of tissues and cells (Fu et al., 2005; Kakoki, Kim, Arendshorst, & Mattson, 2004; Matsuoka et al., 1996; Mirmiran et al., 2016; G Wu & Meininger, 2002) In regards to CO, high arginine intake has been shown to upregulate heme oxygenase 1 expression and increase CO synthesis in endothelial cells as well as nonvascular cells such as VSMC (Li et al., 2009). As mentioned earlier, dietary arginine supplementation has also been show to increase heme oxygenase 3 expression leading to increases in CO within white adipose tissue (WAT) of ZDF rats (Fu et al., 2005) and diet induced obese rats (W. Jobgen, Fu, et al., 2009). Finally, high intake of oral arginine has been shown to stimulate hydrogen sulfide production in both the plasma and lungs of rats with high blood flow induced pulmonary hypertension (Yanfei, et al., 2006).

1.3.4 Maintenance of Gut Health

L-Arg in the gut has been shown to regulate key functions of intestinal health including limiting intestinal alterations and maintenance of intestinal barrier and immune-physiological functions. Arginine's ability to maintain gut health has mainly been attributed to its role as a substrate for arginase and NOS (particularly iNOS) enzymes (Fritz, 2013). Metabolism of L-Arg

via arginase into L-ORN may help enhance epithelial barrier function as L-ORN is a precursor for polyamines (Wang et al., 2009) Polyamines are known to maintain gut health through their involvement in mucosal protection of the gastrointestinal tract and intestinal epithelial migration (Luiking et al., 2012). Additionally, proline can be produced *via* ornithine aminotransferase metabolism of L-ORN. Proline is a precursor for collagen synthesis and thus plays roles in wound healing and cell migration in epithelial cells and fibroblasts (Luiking et al., 2012). NOS enzymes play a variety of roles in the intestine including regulating activation of inflammatory cells such as myeloid and lymphoid cells. Additionally, NO synthesized by iNOS in inflammatory monocytes and dendritic cells acts as a regulatory molecule in the gut by controlling levels of inflammatory cytokines, cell differentiation and survival (Fritz, 2013).

1.3.5 Regulation of Energy and Nutrient Metabolism

FAA can regulate a variety of physiological functions including energy metabolism. Arginine, in particular, has shown an innate ability to regulate molecules and metabolic pathways involved in obesity (McKnight et al., 2010). For example, two studies performed by Fu *et al*. in 2005 and 2007 exhibited enhanced lipolysis resulting in decreased WAT and obesity in ZDF rats following 4 and 10 weeks of dietary L-Arg supplementation, respectively. In another study, dietary L-Arg supplementation was found to not only decrease WAT but enhance skeletal muscle and whole-body glucose disposal (W. Jobgen, Meininger, et al., 2009). These effects may be a result of arginine's ability to regulate gene expression in WAT as discussed earlier as well as arginine activated glucose and long-chain fatty oxidation in the liver, skeletal muscle, and WAT (McKnight et al., 2010). Arginine also appears to have positive metabolic effects in humans. Administration of 2 g of oral arginine per day for 45 days increased high density lipoprotein levels and decreased levels of triglycerides, low density lipoprotein, cholesterol and fasting blood sugar as compared to placebo (Naseh Pahlavani et al., 2014). Oral arginine given to patients with type II diabetes mellitus (T2DM) eating a hypocaloric diet resulted in increased fat reduction as compared to placebo while sparing lean body mass (Lucotti et al., 2006). Finally, arginine has also been found to increase both brown adipose tissue generation and thermogenesis in mammals (Hou et al., 2015).

1.3.6 Involvement in Reproduction and Growth

As an amino acid with multiple metabolic fates arginine is involved in a variety of reproductive and fetal growth processes. In fact, supplementation with arginine has been shown to improve fetal survival and growth rates in many different mammals (Che et al., 2013; Lassala et al., 2010, 2011; Mateo et al., 2007). Improvement of fetal survival *via* arginine supplements has mostly been attributed to the avoidance of intrauterine growth retardation (Cudd et al., 2004). In line with this statement, research shows that a fetus is most vulnerable to growth abnormalities when the maternal diet is deficient in nutrients such as proteins and micronutrients during the peri-implantation period and period of rapid placental development (G Wu et al., 1998). In rats, arginine deficiency has been shown to cause intrauterine growth retardation increase fetal reabsorption and death. Conversely, supplementation with dietary arginine was shown to reverse growth restriction in rat models of intrauterine growth retardation (Vosatka et al.1998).

Benefits of arginine supplementation during pregnancy appear to be due to enhanced production of NO and polyamines (Cudd et al., 2004). As a regulator of endothelial-dependent vasodilation, NO plays an important role in monitoring placental-fetal blood flow and thus the transfer of nutrients and O_2 from mother to fetus (Bird et al., 2003). The importance of NO production *via* arginine supplementation in pregnant women is highlighted in women prone to preeclampsia as they display increased levels of the NOS inhibitor ADMA (Savvidou et al., 2003). In these women dietary arginine supplementation reduced the incidence of developing preeclampsia as compared to placebo (Vadillo-Ortega et al., 2011). Additionally, whole body NO deficiency is associated with intrauterine growth retardation (Hata et al., 1998) as are tissues with arginine transport and reduced eNOS activity and NO synthesis in endothelial cells of the umbilical cord (Casanello & Sobrevia, 2002).

On the other hand, polyamines are crucial in pregnancy especially during the peri-implantation period due to their crucial roles in regulating DNA and thus protein synthesis, scavenging of reactive oxygen species as well as cell proliferation *via* mammalian target of rapamycin signalling and tissue differentiation (Xiaoqiu Wang et al., 2014). In mice, inhibition of placental polyamines reduced placental size and fetal growth (Ishida, Hiramatsu, Masuyama, Mizutani, & Kudo, 2002). Similarly, in sheep, knock down of translation of ODC mRNA resulted in abnormal function and growth of the embryo unless the ADC/AGAT pathway is able to compensate and maintain polyamine levels (Xiaoqiu Wang et al., 2014). As a final point both NO and polyamines are

required for angiogenesis and thus are key players in the maintenance of placental blood supply and growth (Cudd et al., 2004).

Arginine also appears to play an important role in male fertility through enhanced spermatogenesis and motility. As previously mentioned, men fed an arginine-deficient diet exhibited a decrease in both sperm number and motility (L. J. Holt & Albanese, 1944). This same study also showed that the reduction in spermatogenesis could be reversed in these same subjects *via* dietary arginine supplementation (L. J. Holt & Albanese, 1944). Infertile men given oral L-Arg supplements of 0.5 g/day also displayed increased sperm count and furthermore an increase in successful pregnancies of their partner (Tanimura, 1967). Similar results in animal studies have led to the conclusion that L-Arg is required for proper spermatogenesis and motility in male mammals (Chen et al., 2018; Radany & Atherton, 1981).

1.3.7 Involvement in Wound Healing

Research regarding the role of arginine in wound healing has mainly focused on acute wounds such as superficial cuts as compared to chronic wounds such as ulcers. In acute wound healing arginine is sequentially utilized by two metabolic pathways. Initially the NOS pathway is activated during the first 1-3 days post-injury, followed by the arginase pathway in the latter 4-21 days (Stechmiller et al., 2005). All three NOS isoforms are found within various skin cell types where they aid in the first stage of wound healing known as the inflammatory stage. Of the three isoforms, iNOS appears to be of particular importance as arginine supplemented iNOS-knock out mice did not exhibit the enhanced collagen deposition and wound breaking strength of arginine supplemented wild-type mice (Shi, et al., 2000). Furthermore, during the inflammatory phase, iNOS transcription, expression and activity is induced by a wide variety of cytokines leading to increased NO production. The primary role of NO in these early stages of wound healing is to create an environment that is toxic to bacteria and increase blood flow allowing for the infiltration of immune cells. Additionally, NO released from fibroblasts stimulates collagen synthesis (Stechmiller et al., 2005).

Following suppression of the NOS pathway, the arginase pathway is stimulated resulting in the proliferation stage (Stechmiller et al., 2005). Arginase I and II metabolize L-Arg to L-ORN, the precursor of the polyamines putrescine, spermidine and spermine, all of which play roles in DNA replication and thus cell proliferation (Sidney M Morris, 2007). Additionally, L-ORN can be

metabolized into proline, a prominent building block of collagen. Collagen deposition is important in wound healing as structural support as well as support of cell proliferation and differentiation (Stechmiller et al., 2005). Finally, transforming-growth-factor β appears to be involved in regulating the transition between these two pathways as it inhibits iNOS but stimulates arginases and collagen synthesis (Alexander & Supp, 2014).

1.3.8 Role in Immune Function

Arginine metabolism has been found to be intimately involved in both innate and adaptive immunity. As in wound healing, NOS and arginase isoforms are arginine metabolic enzymes that can be regulated to control innate immune response *via* myeloid cells (P. C. Rodriguez, Ochoa, & Al-Khami, 2017). For example, macrophages are classified as M1 or M2 based on which arginine metabolic enzyme is primarily activated *via* pro-inflammatory cytokines. M1 macrophages generally break down arginine *via* NOS enzymes creating a cytotoxic environment for the elimination of pathogens and tumor cells (Bogdan, 2001). As in endothelial cells citrulline is recycled back into arginine *via* the citrulline-NO cycle in order to support the M1 macrophage's demand for arginine (Qualls et al., 2012). Arginase 1 can become upregulated in M1 macrophages to maintain balance and ensure host cytotoxicity does not occur due to excessive NO production (P. C. Rodriguez et al., 2017). In M2 macrophages, arginase 1 is preferentially expressed. These cells appear to play anti-inflammatory and wound healing roles by shunting arginine into pathways which produce ornithine, proline and polyamines.

Arginine also plays roles in acquired immunity through a variety of mechanisms. For example, as a precursor for polyamines, arginine is required for sufficient production of human T-lymphocytes in response to mitogens (Field et al., 2002). Arginine has also been demonstrated to be vital in the maturation of progenitor-B cells to precursor B lymphocytes in murine models (De Jonge et al., 2002). Additionally arginine stimulates growth hormone secretion *via* inhibition of somatostatin (Campbell et al., 2007). In turn, growth hormone increases thymus production of T lymphocytes, response of T lymphocytes to antigens, and the number of hematopoietic progenitor cells. In clinical studies on individuals with burns, human immunodeficiency virus, major traumas and gastrointestinal surgical operations; arginine supplementation has been shown to improve immune function (Calder & Yaqoob, 2013).

1.4 Oral Arginine Supplements and Their Uses

L-arginine supplements are readily available over the counter and commonly taken by the general public in the hopes of ameliorating a wide variety of conditions. L-arginine supplements are also popular among healthy people including athletes for their proposed cardiovascular performance enhancing effects (Maxwell et al., 2001). As such, L-Arg supplements have been documented for use in conditions such as essential hypertension (Lekakis et al., 2002), hypercholesterolemia (Clarkson et al., 1996), angina pectoris (Blum et al., 1999), congestive heart failure (Balderas-Munoz et al., 2012), atherosclerosis (Javanmard et al. 2009), intermittent claudication (Leone et al., 1992), diabetes (Lucotti et al., 2006), obesity (W. Jobgen, et al., 2009), cancer (Ma et al., 2007), ulcers (Liu et al., 2017), burns (De-Souza & Greene, 1998), wound healing (Shi et al., 2000), decreased immune function (Calder & Yaqoob, 2013), female infertility (Battaglia et al., 1999), male infertility (Tanimura, 1967), erectile dysfunction (Chen et al., 2001), preeclampsia (Vadillo-Ortega et al., 2011), enhancing fetal survival (Che et al., 2013; Lassala et al., 2010, 2011; Mateo et al., 2007) and senile dementia (Ohtsuka & Nakaya, 2000). Generally, L-arginine supplements are taken with the hopes of increasing NO production. Thus, NO production has been the focal point of most studies utilizing L-arginine supplements in diseases. However, as L-arginine is metabolized by a wide variety of enzymes this may not be the only potential therapeutic effect. Additionally, alternative methods of altering L-arginine metabolism (i.e. inhibiting enzymes such as arginase) may be more useful as in some cases L-arginine supplements have proved to not only be unhelpful but unsafe (Dioguardi, 2011). The use of arginine in hypertension, hypercholesterolemia and T2DM has been highlighted below. One important thing to note is the wide range of doses and duration of supplementation used in different studies. Thus, doses from 3 to 42 g/day have been reported (Shao and Hathcock, 2008), which provides a reference point or relevance to the doses of 500 mg/kg/day and 1000 mg/kg/day used in our studies in this project.

1.4.1 Hypertension

Hypertension caused by endothelial dysfunction is often due to a decrease in the amount of NO reaching the vascular smooth muscle. This can be caused by a decreased production of NO in the endothelium due to factors such as lack of available arginine, over expression of arginase I/II or competition of ADMA (Förstermann & Sessa, 2012). On the other hand, while sufficient

amounts of NO may be being produced, adequate amounts may not be reaching the vascular smooth muscle due to quenching by superoxide anions (Förstermann & Sessa, 2012). As L-Arg is the substrate used by eNOS it follows that many clinical studies have attempted to use L-Arg supplements to ameliorate hypertension.

The ability of L-Arg supplements to lower blood pressure (BP) appears to follow a trend. When given to healthy individuals or even athletes generally there is not much of a change in BP (Ast et al., 2011; Naseh Pahlavani et al., 2014). However, when given to individuals with disease related hypertension such as obesity, hypercholesterolemia or diabetes there does appear to be beneficial effects on lowering BP or enhancing flow mediated vasodilation (Clarkson et al., 1996; Lekakis et al., 2002; Piatti et al., 2001). For example, supplementing individuals with type II diabetes with 1 g of L-Arg three times a day for one month resulted in a significant decrease in systolic BP while significantly increasing forearm blood flow (Piatti et al., 2001). Likewise, individuals with essential hypertension showed significantly improved flow mediated vasodilation following L-Arg supplementation (Lekakis et al., 2002). Oppositely, in healthy individuals receiving 2 g of L-Arg a day for 45 days did not show any significant changes in systolic or diastolic BP (Naseh Pahlavani et al., 2014).

As arginase is known to steal L-Arg from eNOS and overexpression / and or increased activity of this enzyme has been linked to endothelial dysfunction it has been thought that inhibiting arginase rather than supplementing L-Arg may be a more effective way of treating hypertension. One study on Zucker Obese and Zucker Lean rats found that obese rats had significantly increased arginase activity resulting in significantly lower levels of circulating plasma arginine and nitrate+nitrite (NOx). Treatment with an arginase inhibitor for 4 weeks significantly decreased mean arterial pressure (MAP) while increasing arginine and NOx levels to levels similar to those achieved with 4 weeks of arginine supplementation (Peyton et al., 2018).

1.4.2 Hypercholesterolemia

L-Arg supplements have been shown to have some beneficial effects in individuals with hypercholesterolemia. Interestingly, most studies report an in improvement in flow mediated vasodilation rather than changes in the lipid profile. For example, hypercholesterolemia patients receiving 7 g of L-Arg 3 times a day for 4 weeks showed a significant increase in flow mediated vasodilation with no change in BP, heart rate or the lipid profile (Clarkson et al., 1996). Similar

results were achieved in a study supplementing with 3.3 g of L-Arg a day through a nutrient dense bar eaten twice a day. Flow mediated vasodilation was significantly increased while serum cholesterol was significantly decreased (Maxwell et al. 2000). However, 25 mg of niacin was also present in each bar, as niacin is known to slightly lower low density lipoprotein levels this may be why cholesterol was lowered in this study but not the previous one. Oddly, a previously mentioned study supplementing healthy men with 2 g of oral L-Arg a day for 45 days did show improvements in the lipid profile (Naseh Pahlavani et al., 2014). This is opposite to the trend mentioned above. Interestingly, levels of the L-Arg competitor ADMA appear to have some inverse correlation with success of L-Arg supplementation on vasodilation. For example, another study on asymptomatic hypercholesterolemic patients found that these subjects had ADMA levels >100% higher than the control non-hypercholesteremic subjects. Administration of i.v. L-Arg was able to significantly increase the L-Arg/ADMA ratio and in turn significantly improve endothelium-dependent vasodilation and increase urinary nitrate excretion rates (Böger et al., 1998). Thus individuals with high ADMA levels may be more receptive to L-Arg supplements than those with low ADMA levels (Böger, 2007).

1.4.3 Type 2 Diabetes Mellitus

The association between T2DM and heightened levels of MG has been known for many years (McLellan et al., 1994). MG results in hallmark characteristics of diabetes including endothelial dysfunction (Dhar et al., 2011; Dhar et al., 2012). L-Arg supplements have been proposed to prevent formation of advanced glycation endproducts (AGEs) in T2DM as it is a known MG scavenger and the precursor for NO production. For example, one study on a rat model of T2DM found that administration of 1.5 g/kg/day of L-Arg reduced inflammatory mediators and RAGE-sensitive tissues in the liver and lungs as compared to diabetic control rats (Pai et al., 2010). Another study administered 3 g of L-Arg 3 times a day for a month to diabetic patients and found that treatment significantly increased cGMP levels, forearm blood flow and significantly reduced peripheral vascular resistance as compared to placebo (Piatti et al., 2001). In a second study, L-Arg supplements of 8.3 g/day for 21 days was used as an adjunct with a hypocaloric diet and exercise program to treat T2DM. Patients given L-Arg exhibited significantly decreased fasting glucose levels after 2 weeks as compared to the placebo group. The L-Arg group also showed significant reductions in systolic and diastolic blood pressure, insulin levels and endothelin-1 with increases

in cGMP and NOx as compared to placebo (Lucotti et al., 2006). This study provides evidence that L-Arg added to a healthier lifestyle may be beneficial in alleviating symptoms of T2DM.

A few studies have also looked at the efficacy of L-Arg in treating other symptoms of diabetes including neuropathy and nephropathy. Diabetic rats given 2.58 g/L of L-Arg *via* drinking water for 3 weeks did not experience the diabetes related hyperalgesia or allodynia that the non-supplemented diabetic rats did (Rondón et al., 2018). In a study assaying the effect of 6.05 g/kg/day of L-Arg in diabetic mice, no significant improvement of diabetes related nephropathy was observed (You et al., 2014). Diabetes related retinopathy is believed to be caused by over active arginases and thus providing more substrate *via* L-Arg supplementation may actually worsen the condition (Caldwell, Zhang, Romero, & Caldwell, 2010).

1.5 Animal and Human Studies on Physiological Impact

The physiological impact of L-Arg supplements on the four main arginine metabolic pathways has not been widely studied. The impact of D-Arg has been studied even less as it is generally believed to be inert in humans and functions more in bacteria (Cava et al., 2011). Understanding the effects of both L- and D-Arg supplements on these pathways is crucial for determining the safety of these supplements, especially because deficiencies of arginine metabolizing enzymes are associated with diseases such as hyperammonemia (Crombez & Cederbaum, 2005), endothelial dysfunction (Cooper, 2004; Dhar et al., 2012), CNS (Maestri et al., 1999) and muscle dysfunction (Maestri et al., 1999).

1.5.1 Impact on eNOS Pathway

Of the four arginine metabolic pathways, the NOS-NO pathway is potentially the most studied as it is the pathway responsible for producing NO. The effects of L-Arg supplements on eNOS expression have been controversial. Generally, an increase in eNOS expression is observed following L-Arg supplementation. For example, increased eNOS expression was observed in the aorta of hypercholesterolemic rabbits (Javanmard et al., 2009) and Wistar rats with pulmonary embolism (Zhang et al., 2006). Interestingly, in cultured human umbilical vein cells (HUVECs) incubated with L-Arg an increase in activity but not expression was observed at 2 hours, however, 7 days later eNOS expression and activity were decreased (Mohan et al., 2012). Similarly, NO production, as indicated by NOx or nitrite/nitrate levels, is increased by L-Arg in some studies

(Kohli et al., 2004) but unchanged in others (Alvares et al., 2012). Similar to how L-Arg supplements only appear to lower blood pressure in a disease state, eNOS expression and NO production only appear to be increased in studies using disease models. The effect of D-Arg on the NOS-NO pathway has not been reported.

1.5.2 Impact on Arginase Pathway

As overactive arginase enzymes can lead to endothelial dysfunction most studies focus on the inhibition of arginase rather than supplementing with L- or D-Arg. However, one study using HUVECs found that while acute (30 min) administration of L-Arg did not affect arginase II expression or activity, chronic administration (7 days) enhanced both expression and activity. Another study on ageing Wistar rats found that L-Arg supplementation did not change arginase activity in any tissues except the aorta where it was decreased and the lung where it was increased (Moretto et al., 2015). The effect of L-Arg on urea levels has also been assayed in a few studies. One study fed Wistar rats a high arginine diet with a dose approximately equivalent to 25 g/day in humans. These rats had significantly increased plasma urea levels compared to the control group (Holecek & Sispera, 2016). Interestingly, a study looking at the effect of a one-time 3 g dose of L-Arg on human salivary urea found that urea levels were not significantly different between the L-Arg and placebo group (Vuletic et al., 2013). The effect of D-Arg on the arginase pathway was not reported.

1.5.3 Impact on Arginine Decarboxylase Pathway

The ADC pathway has mainly been studied in regards to its role in reproduction and gut health. However, very few studies have reported the effect of L-Arg supplements on this pathway. Based on a survey of the literature, no studies have reported the effect of L-Arg supplements on expression or activity of ADC or agmatinase. One study on mice with irritable bowel syndrome found that mice fed a high L-Arg diet did not have altered colon tissue polyamine content (K. Singh et al., 2019). Another study on diabetic rats found that those supplemented with 2.58 g/L of L-Arg for three weeks had significantly increased agmatine levels as compared to supplemented rats (Rondón et al., 2018). Information on the effect of D-Arg on these pathways could not be found.

1.5.4 Impact on Arginine:Glycine Amidinotransferase Pathway

The physiological impact of arginine supplements on the AGAT pathway has not been well studied. A study in 2009 investigated the effect of a 9 g dose of L-Arg for 4 days in individuals with coronary artery disease (Jahangir et al., 2009). This study revealed that L-Arg significantly increased plasma levels of guanidinoacetate, the precursor for Cr. Interestingly, Cr levels were unchanged by L-Arg. Unfortunately, the expression and activity of AGAT and GAMT were not assayed in this study. The effect of D-Arg on the AGAT pathway in mammals has not been reported.

1.6 Methylglyoxal

MG is a highly reactive α dicarbonyl aldehyde which can be produced through a variety of metabolic reactions but is most commonly produced *via* fructose and glucose metabolism (Matafome et al. , 2017). MG is known by many names including, pyruvaldehye, pyruvic aldehyde, 2-Oxopropanal and acetylformaldehyde. It is a yellow, somewhat viscous liquid with a pungent smell and molecular weight of 72.063 g/mol (Fig. 1.3).

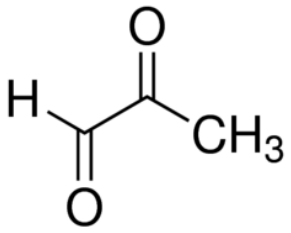


Figure 1.3. Chemical structure of methylglyoxal.

(Image source: https://www.sigmaaldrich.com/catalog/product/sigma/m0252?lang=en&region=CA)

1.6.1 Sources of Methylglyoxal:

MG can be produced *via* enzymatic and non-enzymatic reactions which occur during glucose, lipid and amino acid metabolism. Enzymatically, MG is produced by three enzymes including MG synthase; cytochrome P450 IIE1 isozyme(s) and amine oxidase(s) (Fig. 1.4, Kalapos, 1999). The first enzyme, MG synthase, produces MG during glycolytic bypass. MG synthase metabolises dihydroxyacetonephosphate to MG and phosphate (Thornalley, 1996). The second enzyme, cytochrome P450 IIE1 isozyme, converts acetone derived from lipid metabolism into MG *via* a

two-step process in which acetol is an intermediate (Kalapos, 1999). Thirdly, semicarbazide-sensitive amine oxidases, participate in amino acid metabolism and can form MG *via* oxidation of aminoacetone formed during threonine and glycine catabolism (Kalapos, 1999). Non-enzymatically, MG is primarily produced *via* the fragmentation of triphosphates. In physiological systems this most commonly occurs *via* the removal of a phosphate group from dihydroxyacetonephosphate or glyceraldehyde-3-phosphate (Fig. 1.4, Thornalley, 1993). This process occurs under euglycemic conditions but is increased with the accumulation of metabolites such as fructose, glucose, acetone, acetol, dihydroxy-acetone and D-glyceraldehyde which enhance the flux of triose-phosphates (Kalapos, 1999; Thornalley, 1996).

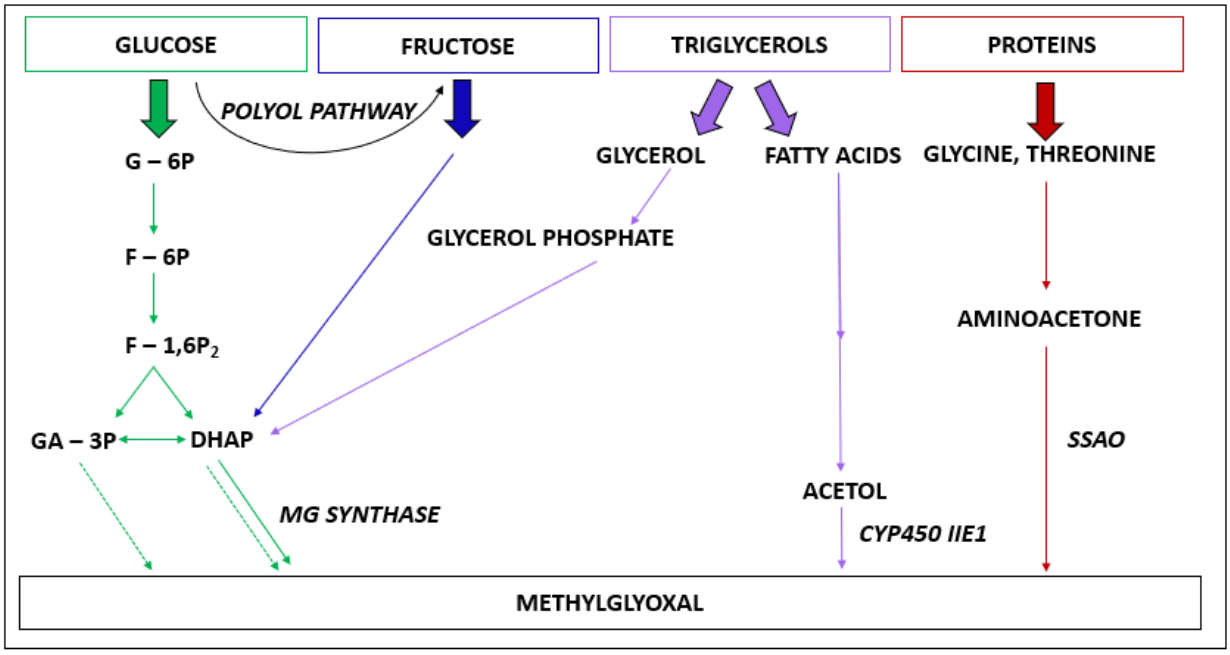


Figure 1.4. Sources and synthesis of methylglyoxal.

Methylglyoxal is primarily produced from the non-enzymatic fragmentation of glucose and fructose derived triosephosphates. Other minor routes of methylglyoxal synthesis include the enzymatic breakdown of dihydroxyacetonephosphate, fatty acid derived acetol and amino acid derived aminoacetone. Note: dotted line represents non-enzymatic production of methylglyoxal. Abbreviations: CYP450 IIE1 - cytochrome P450 IIE1 isozymes and amine oxidase; DHAP– dihydroxyacetonephosphate; F-1,6P_2 – fructose 1,6-bisphosphate; F-6P – fructose 6-phosphate; G-6P – glucose 6-phosphate; GA-3P – glyceraldehyde 3-phosphate; MG – methylglyoxal; SSAO - semicarbazide-sensitive amine oxidases.

1.6.2 Degradation of MG: The Glyoxalase System

MG is degraded by the glyoxalase system in the cytosol of cells. This system consists of two enzymes, glyoxalase I and II, which are aided by reduced glutathione (Fig. 1.5, Thornalley, 1996). Glyoxalase I is found within the cytosol of all eukaryotic cells. However, its specific activity in humans is highest in the pancreas, lung, kidney and brain and lowest in adipose tissue and liver. Glyoxalase II is also found within all eukaryotic cells and is located in the cytosol and mitochondria (Thornalley, 1993). In short, hemthioacetal which is formed non-enzymatically from MG and reduced glutathione is catalysed to S-D-lactoylglutathione by glyoxalase I. Glyoxalase II then hydrolyzes S-D-lactoylglutathione to inert D-lactate and the reduced glutathione lost in the previous reaction is restored (Fig. 1.5, Thornalley, 1996).

D-lactate can be further metabolised to pyruvate by 2-hydroxyacid dehydrogenase (Fig. 1.5). It should be noted that D-lactate is also produced in the gastrointestinal tract by bacteria such as lactobacilli which is then usually metabolised to acetate and other short chain fatty acids by other intestinal bacteria (Ewaschuk et al.,2005). D-lactate can be transported through cell walls by three different mechanisms: the specific lactate transporter, the inorganic anion exchange system and by non-ionic diffusion (Thornalley, 1993). Finally, D-lactate is excreted in the urine and rapidly reabsorbed from the renal filtrate *via* the renal tubules. Excretion of D-lactate can also occur through the feces or sweat (Thornalley, 1993).

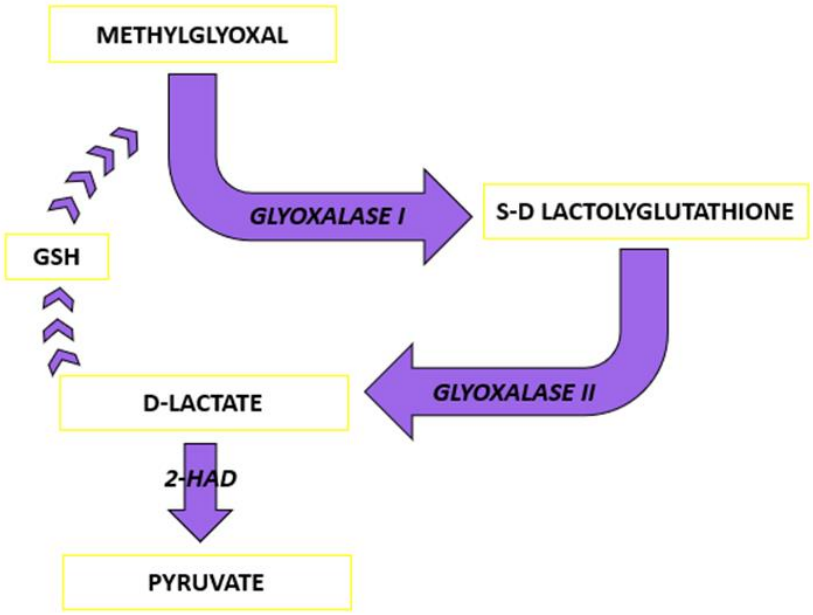


Figure 1.5. A schematic diagram of methylglyoxal-glyoxalase metabolic pathway.
Methylglyoxal is detoxified into inert d-lactate *via* the glyoxalase system. This system involves two enzymes and reduced glutathione (GSH) which is regenerated. D-lactate can be further broken down by 2-hydroxyacid dehydrogenase (2-HAD).

1.6.3 Physiological Levels of Methylglyoxal

MG is a highly reactive molecule which is estimated to be 99% bound to proteins and other biomolecules leaving only 1% of MG in the body in its free form (Chaplen, Fahl, & Cameron, 1998). Due to MG being so highly bound, accurately estimating its levels in the body can be difficult and most likely will result in estimates lower than the actual amount of MG present. For example, in one study healthy human subjects had 0.332 ± 0.062 µM of free plasma MG (Turk, et al., 2008). Another study reported similar results indicating healthy human subjects to have a mean free MG plasma level of 0.556 ± 0.15 µM (Kong et al., 2014). In healthy SD rats the amount of MG detected in the serum was 1.81 ± 0.41 µM (Jia & Wu, 2007) while another study reported a much higher MG plasma level of 11.2 ± 0.44 µM in Wistar Kyoto rats (WKY, Wang et al., 2004). When comparing the amount of MG present in different organs/tissues of SD rats it was found that the aorta had much higher levels than any of the other organs or tissues assayed (Wu et al., 2010).

1.6.4 Methylglyoxal Induced Advanced Glycation End Products

Advanced glycation end products or AGEs are a complex group of compounds formed when reducing sugars and amine residues on proteins, nucleic acids or lipids react non-enzymatically. Highly reactive intermediate carbonyl groups such as α-dicarbonyls like MG are major contributors to AGEs production *in vivo* (Goh & Cooper, 2008). The formation of AGEs *via* the Maillard reaction begins when the amine group of a protein is condensed by the reducing sugar at the N-terminal or an arginine or lysine side chain (Fig. 1.6). This results in the carbonyl group of the sugar attaching with the protein to form an unstable intermediate known as a Schiff base (Fig. 1.6, Richard Bucala & Cerami, 1992). The formation of the Schiff base is very fast and readily reversible, however, over time the Schiff base will undergo an Amadori rearrangement resulting in the formation of a stable Amadori product which is cyclized into a ring structure. Schiff bases and Amadori products are known as "early glycation products" as eventually the non-enzymatic oxidation, degradation or rearrangement of the Amadori product results in irreversible AGEs (Fig. 1.6, Richard Bucala & Cerami, 1992). As a result of the Maillard reaction AGEs can be broadly categorized as brown and fluorescent cross-linking substances, non-fluorescent cross-linking products or non-fluorescent, non-crossing adducts (Sharma et al., 2015).

Originally, glucose was thought to be the primary sugar involved in AGE formation. However, a study showed that MG is actually the main culprit in AGEs formation. Interestingly, MG, formed in the early stages of the Maillard reaction can directly bind non-enzymatically to the amino residues of proteins in an oxidation reaction that bypasses the formation of Schiff bases and Amadori products to produce AGEs (Shinohara et al., 1998). MG is highly reactive with arginine, lysine and cysteine residues of proteins resulting in the formation of a wide variety of AGEs (Fig. 1.6). When binding with arginine residues MG tends to form cyclic imidazoline adducts. A study by Klopfer *et al*. (2011) revealed eight compounds which were produced when MG and L-Arg were co-incubated in a phosphate buffer. However, only the following six were successfully isolated using HPLC: N^{δ}-(4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydropyrimidine-2-yl)-l-ornithine (MG-H1), N^{δ}-(5-methyl-4-oxo-5-hydroimidazolon-2-yl)-l-ornithine (MG-H2) of 2-amino-5-(2-amino-4-hydro-4-methyl-5-imidazolon-1-yl) pentanoic acid (MG-H3), diastereomeric aldol addition products of MG-H3 and formaldehyde, argpyrimidine and N^{7}-carboxyethylarginine (Klöpfer et al., 2011). This study revealed that MG-H3 is the key intermediate in the reaction between MG and L-arginine. pH induced ring opening reactions were found to be a key determinant

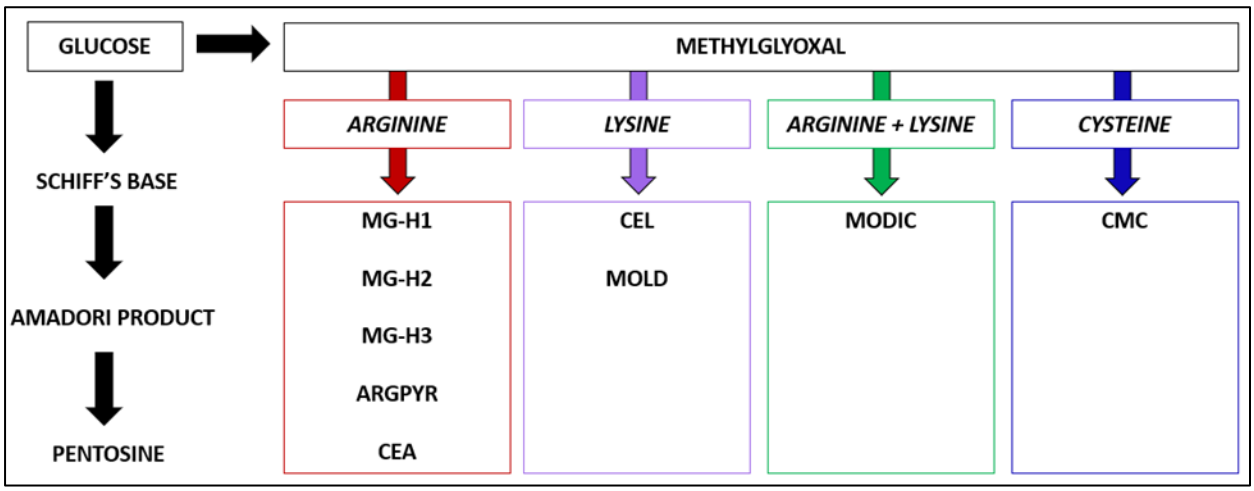


Figure 1.6. A schematic diagram of advanced glycation end products (AGEs) generated from methylglyoxal.

Methylglyoxal is a major contributor to AGE formation. Methylglyoxal reacts with the amino acid residues of proteins to produce AGEs. Methylglyoxal reacts with arginine residues to form N^{δ}-(4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydropyrimidine-2-yl)-l-ornithine (MG-H1), N^{δ}-(5-methyl-4-oxo-5-hydroimidazolon-2-yl)-l-ornithine (MG-H2) of 2-amino-5-(2-amino-4-hydro-4-methyl-5-imidazolon-1-yl) pentanoic acid (MG-H3), argpyrimidine (ARGPYR) and N^{7}-carboxyethylarginine (CEA, Klöpfer et al., 2011). When methylglyoxal reacts with lysine residues N^{ε}-carboxyethyl-lysine (CEL) and the imidazolium cross-link 2-ammonio-6-[1-(5-ammonio-6-oxido-6-oxohexyl)-5-methylimidazolium-3-yl] hexanoate (MOLD) is formed (Nasiri et al., 2011). A dimer crosslink between arginine and lysine produces methylglyoxal-derived imidazolium cross-link (MODIC, Nasiri et al., 2011) and finally reacting with cysteine produces s-carboxymethyl-cysteine (CMC, J. Zeng & Davies, 2005).

of which compound was preferentially formed with MG-H1, MG-H2 and argpyrimidine being the most stable. At a physiological pH of 7.4, argpyrimidine was the only MG derived AGE that accumulated over time as all the other five compounds would quickly degrade after formation (Klöpfer et al., 2011). MG is also known to bind to the ε amino group of lysine residues to form N^{ε}-carboxyethyl-lysine (CEL) and the imidazolium cross-link 2-ammonio-6-[1-(5-ammonio-6-oxido-6-oxohexyl)-5-methylimidazolium-3-yl] hexanoate (MOLD). When MG reacts with both

arginine and lysine residues the resultant structure MG-derived imidazolium cross-link (MODIC) is formed (Nasiri et al., 2011). Finally, MG can bind to the thiol group of cysteine forming the irreversible adduct s-carboxymethyl-cysteine (J. Zeng & Davies, 2005).

AGEs tend to exert their pathological effects by either mutating proteins or interacting with AGE receptors including AGE-R1, AGE-R2, AGE-R3 and receptor for advanced glycation end products (RAGE). Proteins such as collagen and laminin, which make up the extracellular matrix, can be easily glycated by AGEs. As these proteins tend to have a slow turnover rate, crosslinking them with AGEs promotes fibrosis and decreases connective tissue flexibility which can lead to arterial stiffness and eventually endothelial dysfunction (Cooper, 2004). It has also been theorized that AGEs inhibit NO activity leading to a disruption in vasodilation, increased blood pressure and ultimately endothelial dysfunction. A few different mechanisms have been proposed including the degradation of eNOS mRNA by AGEs (Rojas et al., 2000), the quenching of NO by AGEs (Bucala et al., 1991), or decreased phosphorylation of eNOS leading to lower enzyme activity as a consequence of AGEs binding to the RAGE receptor (Xu et al., 2003).

As mentioned above AGEs can act on four receptors, however, AGEs primarily cross link with the RAGE receptor. RAGE mediated signalling has been found to primarily occur *via* extracellular receptor kinase 1/2, p38 and c-Jun N-terminal kinases activation (Bierhaus et al., 2005). When AGEs bind RAGE the pro-inflammatory transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is activated. When activated NF-κB binds to DNA, it results in transcription of NF-κB regulated target proteins including pro-inflammatory cytokines (IL-1α, IL-6, tissue necrosis factor α), adhesion molecules (vascular cell adhesion molecule-1), pro-thrombotic (tissue factor, thrombomodulin) and vasoconstrictive (endothelin-1) mediators (Bierhaus et al., 2005). Additionally, NF-κB increases expression of RAGE. Thus, prolonged activation of NF-κB results in upregulation of RAGE creating a vicious cycle of sustained NF-κB signalling and inflammatory response. Finally, ligation of RAGE with AGEs results in decreased reduced glutathione which hinders glyoxalase 1 recycling. Insufficient recycling of glyoxalase 1 reduces the ability of the glyoxalase system to detoxify MG leading to increased MG in the body and subsequently increased AGE production and oxidative stress (Bierhaus et al., 2005).

1.6.5 Methylglyoxal Induced Pathology

Increased MG levels have been observed in many different disease states including, Alzheimer's (Angeloni et al., 2014), Parkinson's (Xie et al., 2014), obesity (Jia et al., 2012), cancer (Nokin et al., 2019), heart failure (Papadaki et al., 2018), atherosclerosis (van Eupen et al., 2013), endothelial dysfunction (Cooper, 2004; Dhar et al., 2012), essential (Sudesh Vasdev & Stuckless, 2010) and disease state related hypertension (Dhar et al., 2014) and diabetes (Dhar et al., 2011) Furthermore, increases of MG in these diseases have been positively correlated to hallmark characteristics of the aforementioned diseases. The toxicity of MG depends on the amount present in the body. For example, in healthy individuals the amount of MG present (usually, < 1 µ M) (Kong et al., 2014; Turk et al., 2008) is easily handled by the glyoxalase system. Unfortunately, MG tends to accumulate in disease states. This becomes problematic as MG is known to be cytotoxic as it can bind to the guanyl residues of DNA resulting in apoptosis (Thornalley, 1998). Additionally, as mentioned above, MG is a known precursor of AGEs which contribute to the aging process, inflammation and free radical production. Additionally, MG itself is known to contribute to oxidative stress (L. Wu & Juurlink, 2002). The role of MG in hypertension and T2DM has been highlighted below.

1.6.6 Methylglyoxal and Hypertension

MG has been associated with both, essential hypertension (Wang et al.,2005) as well as hypertension secondary to diseases such as T2DM (A. Dhar et al., 2011a). Studies have shown that MG accumulates in VSMCs (L. Wu & Juurlink, 2002) and vascular endothelial cells (Shinohara et al., 1998) where it can wreak havoc on the ability of the associated blood vessels to dilate. For example, incubation of SD rat aortic rings with 100 µM of MG lead to significantly decreased endothelium-dependent vasodilation. This decrease in vasodilation was accompanied by a significant increase in MG levels in both the VSMCs and aorta. Additionally, incubating SD rat aortic rings with 25 mM of glucose significantly hindered endothelium-dependent vasodilation and significantly increased cellular and aortic MG levels. Both, most likely a consequence of the increased production of MG *via* increased flux through the glycolytic pathway (Dhar et al., 2012). Interestingly, incubation of both HUVECs and VSMCs with MG also significantly increased the expression and activity of arginase I and II (Dhar et al., 2012). As mentioned above, both arginase isoforms are known to outcompete L-Arg from eNOS which has been linked to endothelial

dysfunction (Johnson, 2004; Rodriguez et al., 2000; Romero et al., 2008; Shin et al., 2012; Zhang et al., 2004). Incubation of HUVECs and VSMCs with MG also resulted in increased expression of the pro-inflammatory transcription factor NF-κB, reactive oxygen species (ROS) and the MG-derived AGE, CEL (Dhar et al., 2012). Similarly, VSMCs of hypertensive rats also showed increases in MG, AGEs, NF-κB expression and oxidative stress in addition to reduced activity of glutathione synthase (L. Wu & Juurlink, 2002). Accumulation of reactive oxygen species can be problematic as they are known to quench the NO required for endothelium-dependent vasodilation. Additionally, as mentioned above, AGEs can glycate extracellular matrix proteins leading to arterial stiffness (Cooper, 2004) as well as activate RAGE causing an outflow of pro-inflammatory and vasoconstricting mediators, increase expression of RAGE and deplete antioxidants such as reduced glutathione (Bierhaus et al., 2005). RAGE activation has also been associated with NF-κB mediated increased levels of angiotensin and the AT_1 receptor, both of which contribute to vasoconstriction (Dhar et al., 2014). Finally, chronic administration of MG to SD rats resulted in increases in blood pressure, the renin-angiotensin-aldosterone system and circulating catecholamines (Dhar et al., 2014).

Many studies investigating the link between MG and hypertension typically do so under a hyperglycemic condition. However, this link has also been demonstrated under euglycemic conditions using spontaneously hypertensive rats (SHR) and WKY control rats. Wang et al. (2005) found that blood pressure was age dependently increased in SHR *vs* WKY rats. This increase in blood pressure was associated with a significant increase in plasma and aortic MG levels in SHR but not WKY rats. SHR also had significantly increased levels of CEL and CML *vs* WKY rats. As neither model exhibited hyperglycemia and glucose and insulin levels in SHR rats were not significantly different than WKY rats; it was concluded that MG can accumulate and cause hypertension even under euglycemic conditions (Wang et al., 2005). It was hypothesized that excess production of MG from other sources such as amino acid metabolism *via* semicarbazide-sensitive amine oxidase was the cause accumulation of MG (Wang et al., 2005).

Finally, in humans, an epidemiological study interested in the relationship between plasma MG levels in individuals with Type 1 diabetes and cardiovascular events found that increases in glucose levels were positively correlated with increases in MG and CML. Additionally, those with higher MG levels had significantly higher low-grade inflammation and incidences of endothelial dysfunction (Hanssen et al., 2017). A similar study with T2DM patients with a 10 year follow up

found that individuals who had experienced a cardiovascular event or had died of one prior to follow up had significantly higher baseline AGEs levels than those who did not (Nin et al., 2011).

1.6.7 Methylglyoxal and Type 2 Diabetes Mellitus

As discussed, MG is a metabolite of glucose and fructose metabolism. T2DM is characterized by insulin resistance resulting in a state of chronic hyperglycemia. Thus, it follows that individuals with T2DM will experience accelerated production of MG. For example, newly diagnosed T2DM patients had plasma MG levels that were ~150% higher than aged matched control subjects (Kong et al., 2014). Another study found even greater increases with plasma MG levels increasing ~200% in patients with T2DM (3.6 µM) *vs* healthy controls (1.4 µM, (McLellan et al., 1994). With accumulation of MG comes increased non-enzymatic protein glycation and thus increased AGEs production. Fitting with this statement, diabetic patients have also showed increased AGEs production. For example, serum levels of hydroimidazolone were significantly increased in individuals with T2DM as compared to controls (Kilhovd et al., 2003). Accumulation of MG and MG derived AGEs in the plasma and various tissues/organs has been associated with hallmark characteristics of diabetes including impaired glucose tolerance, insulin resistance (Dhar et al. 2011; L. Wu, 2008), endothelial dysfunction (Dhar et al., 2012), neuropathy (Bierhaus et al., 2012), nephropathy (Giacco et al., 2014) and retinopathy (Fosmark et al., 2006).

The development of hallmark characteristics of T2DM *via* MG has been demonstrated in a study by Dhar *et al*. (2011). SD rats treated with MG showed significantly increased MG levels in their plasma, pancreas, skeletal muscle and adipose tissue as compared to controls. This increase in MG was also associated with a significant increase in fasting glucose, loss in glucose tolerance and decreased plasma insulin levels. The increase in fasting glucose may have been a result of a breakdown in the insulin signalling pathway, a theory that was supported by decreased phosphorylation of insulin-receptor substrate-1 and phosphoinositide 3-kinase activity. Additionally, there was a significant decrease in insulin stimulated glucose uptake and expression of the glucose transporter-4 (GLUT4) receptor in adipose tissue. MG treated rats also exhibited significantly less pancreatic insulin content and glucose-stimulated insulin release as compared to controls. This appeared to be caused by significant reductions in expression of the GLUT2 transporter as well as mRNA and expression positive regulators of insulin gene transcription. Additionally, MG treatment significantly increased expression and mRNA of a negative regulator

of insulin gene transcription. Finally, treatment of a pancreatic β cell line with MG resulted in significantly increased production of ROS and AGEs as well as increased apoptosis, all of which contributed to a significantly decreased insulin secretion.

Increased levels of MG have also been linked to peripheral diabetic neuropathy. For example, it has been found that MG can exert posttranslational modifications on the nociceptor-specific sodium channel Nav1.8 which leads to hyperexcitability of the sensory neurons resulting in hyperalgesia (Bierhaus et al., 2012). MG has also been implicated in diabetic nephropathy. One study knocked out glyoxalase I in mice which resulted in MG levels comparable to those in diabetes (Giacco et al., 2014). This increase in MG resulted in alterations in glomerular proteins and increased oxidative stress leading to kidney pathology (increased AGEs, albumin excretion, mesangial expansion, and glomerular basement membrane thickness) mirroring that observed in the diabetic kidney. Interestingly, overexpression of glyoxalase I in diabetic mice completely avoided signs of diabetic nephropathy (Giacco et al., 2014). Finally, increased serum hydroimidazolone (a MG-derived AGE) has been linked to diabetic retinopathy (Fosmark et al., 2006).

1.7 Methylglyoxal Scavengers

1.7.1 Aminoguanidine

Aminoguanidine is a highly reactive nucleophilic reagent that can bind to MG and produce substituted 3-amino-1,2,4-triazine derivatives (Lo et al., 1994). By scavenging MG, aminoguanidine can prevent the production of MG-derived AGEs. Aminoguanidine can also inhibit semicarbazide-sensitive amine oxidases, thus preventing the production of MG from acetone (Lo, Selwood, et al., 1994). Finally, aminoguanidine can decrease oxidative stress by inhibiting peroxynitrite and iNOS (Szabó et al., 1997). For example, treatment of SHR rats with aminoguanidine significantly decreased MG, MG-derived AGEs and free radicals while increasing glutathione. This resulted in the restoration of endothelium-dependent vasodilation and prevention of pathological changes to the vascular morphology (Dhar et al., 2010). In a diabetic model, treatment with aminoguanidine reduced cardiac hypertrophy and arterial stiffening (Chang et al., 2006). However, due to its high reactivity, aminoguanidine is not recommended for AGEs prevention at concentrations over 500 μM (Lo et al., 1994).

1.7.2 Metformin

Metformin is a diamino biguanide compound traditionally used as a plasma glucose lowering agent. As it is a guanidine compound it can bind and scavenge α-dicarbonyl compounds such as MG. Through this binding, the toxic effects of MG and the production of AGEs can be reduced (Beisswenger et al., 1999). For example, treatment of diabetic patients with metformin resulted in significantly decreased MG levels and improved glycemic control. Reduction of MG as a result of metformin treatment occurred in a dose-dependent manner (Beisswenger et al., 1999). Metformin has also been shown to inhibit glycation resulting in reduced AGEs formation in the lens, kidney and sciatic nerve of diabetic rats (Tanaka et al., 1999).

1.7.3 Alagebrium (ALT-711)

Alagebrium, formerly known as ALT-711, was being developed by Alteon Inc. as an AGE breaker. It is the 4,5-dimethylthiazolium derivative of *N*-phenacylthiazolium bromide. Alagebrium was tested in numerous clinical trials (mainly for heart failure and hypertension) yet never made it past phase II trials supposedly due to financial complications (Fearnley & Lees, 1991). For example in the DIAMOND clinical trial, alagebrium treatment resulted in significantly decreased left ventricular mass and improved diastolic function in patients with diastolic heart failure (Little et al., 2005). In another clinical trial, alagebrium proved effective in improving arterial compliance of aged patients with vascular stiffness (Kass et al., 2001). Most studies, like the two mentioned, mainly focused on the effectiveness of chronically administered alagebrium on AGEs formation. A study by Dhar *et al*. (2010) chose to investigate the acute effect of alagebrium on MG. This study revealed that pre-treatment with alagebrium reduced plasma, urine and tissue MG levels in rats treated with MG. Additionally, alagebrium corrected impaired glucose tolerance and enhanced insulin stimulated glucose uptake in MG treated rats. Levels of reduced glutathione were also increased in alagebrium treated rats (Dhar et al., 2010). This study demonstrated that alagebrium not only breaks AGEs but can prevent toxic effects produced by their precursor MG.

1.7.4 N-acetyl Cysteine

As mentioned above, MG has an affinity for cysteine making cysteine containing compounds effective MG scavengers. As such, N-acetyl cysteine has been found to be a potent MG scavenger

(Jia & Wu, 2007; Vasdev et al., 1998). For example, co-incubating HUVECs and rat aortic endothelial cells (RAECs) with MG and N-acetyl cysteine prevented the increases in MG, decreases in NO production and increased oxidative stress observed with MG incubation alone.

1.8 The Potential of L- and D-Arginine to scavenge methylglyoxal

MG is known to be highly reactive with arginine residues. Thus, it is not surprising that L-Arg has a high affinity (Ka of 1.9 $x10^{-3}$) for MG (Lo et al., 1994). This affinity has proven L-Arg to be an effective scavenger of MG *in vitro* (Dhar et al., 2012). Additionally, D-Arg while thought to be metabolically inert in respect to L-Arg metabolic pathways, is also an effective scavenger of this reactive molecule. It is proposed that MG readily binds to the guanidine group of L and D-Arg (Lo et al., 1994). In fact, when co-incubated with MG, L- and D-Arg bound almost 50% of MG within the first 15 minutes (Dhar et al., 2012).

Furthermore, the incubation of L- and D-Arg in cell cultures has been shown to prevent the pathological effects of MG. For example, L-and D-Arg restored endothelium-dependent vasodilation in RAECs incubated in MG or high amounts of glucose. Interestingly, only L-Arg but not D-Arg prevented N(ω)-nitro-L-arginine methyl ester mediated inhibition of endothelium-dependent relaxation (Dhar et al., 2012). This indicates that while L-Arg may prevent MG and high glucose induced reduced relaxation *via* eNOS-dependent and -independent actions, D-Arg may do so through eNOS-independent reactions alone (Dhar et al., 2012). L-and D-Arg also prevented the high glucose mediated increase in MG levels in VSMCs and RAECs. Furthermore, L-and D-Arg attenuated MG induced increases in NF-κB, oxidative stress and formation of the MG derived AGE, CEL (Dhar et al., 2012). It was also found that MG and high glucose increased arginase expression and activity. Increased expression was attenuated by L-and D-Arg. Oppositely, arginase activity was further increased by the addition of L-and D-Arg (Dhar et al., 2012).

Thus, while L-and D-Arg show promise as MG scavengers their physiological effects on the body and metabolic pathways which utilize L-Arg as a substrate must be further analyzed before the safety of arginine supplements can be confirmed. Furthermore, the effect of D-Arg must be closely analyzed. If it is truly inert in respect to arginine metabolic pathways, D-Arg would be an ideal scavenger. However, as D-Arg was also able to increase arginase activity (Dhar et al., 2012) the supposed inertness of D-Arg requires further investigation.

1.9 RATIONALE FOR STUDY

Oral L-Arg supplements are available over the counter and have been used in many studies in the hopes of ameliorating a wide range of disease states (Clarkson et al., 1996; W. Jobgen, Meininger, et al., 2009; Lekakis et al., 2002; Lucotti et al., 2006; Ohtsuka & Nakaya, 2000). However, currently no scientifically backed minimum or maximum daily dose exists. Furthermore, many of these studies only investigate the effect of a one time or short-term L-Arg treatment. In fact, most studies do no extend beyond a 4-week treatment period (L. J. Holt & Albanese, 1944; Jahangir et al., 2009; Lucotti et al., 2006; Mohan et al., 2012; Rondón et al., 2018) with the exception of a 45 day study by Pahlavani (2017). This makes understanding the pharmacodynamic properties of L-Arg difficult. Thus, we chose to investigate the effect of both a short term (4 week) and long term (16 week) treatment of L-Arg in healthy SD rats. The two doses of 500 mg/kg/day and 1000 mg/kg/day were chosen as they fall on both the low and high end of the dose spectrum reported for rats in the literature (Fu et al., 2005; Matsuoka et al., 1996; Vosatka et al., 1998) and humans (Ast et al., 2011; Clarkson et al., 1996). On the other hand, D-Arg is thought to be metabolically inert in respect to mammalian arginine metabolic pathways (Morris et al., 1997; Palmer et al., 1988) and has therefore been neglected in studies utilizing arginine supplements. Thus, we also sought to understand if this isomer truly has no effect on arginine metabolic pathways. As previously mentioned, arginine is metabolized by four metabolic pathways. However, the majority of studies focus on the NOS and arginase pathways as they are thought to be the most associated with disease states (Alvares et al., 2012; Holecek & Sispera, 2016; Mohan et al., 2012; Moretto et al., 2015; Vuletic et al., 2013; J. Zhang et al., 2006). Thus, we wanted to address this gap in knowledge by investigating the effect of L- and D-Arg supplements on the AGAT and ADC in addition to the NOS and arginase pathways. By assaying all four pathways we aim to better understand both the safety and efficacy of oral arginine supplements.

MG is a toxic molecule found to be heightened in disease states such as T2DM and hypertension. Past research in our lab has found L- and D-Arg to be effective scavengers of MG *in vitro* (Dhar et al., 2012). Thus, we wanted to investigate the ability of L- and D-Arg to scavenge MG *in vivo*. To do so we examined the effect of oral L- and D-Arg supplements on basal MG levels in both a healthy model (SD rats) and a disease model (ZDF rats). To create a complete picture, we also sought to understand the effect of these supplements on the glyoxalase pathway which is responsible for the detoxification of MG to D-lactate. As D-Arg is thought to be metabolically inert

we were keenly interested in its ability to scavenge MG as its supposed inertness would make it an ideal *in vivo* scavenger. Finally, the effectiveness of oral arginine supplements tend to follow a trend in which efficacy is only observed in disease states (Clarkson et al., 1996; Lekakis et al., 2002; Lucotti et al., 2006) but not healthy subjects (Ast et al., 2011; Naseh Pahlavani et al., 2014), especially its effect on blood pressure. By treating both healthy SD rats and obese diabetic ZDF rats we may be able to shed more light on this phenomenon.

1.10 HYPOTHESES AND OBJECTIVES

1.10.1 Hypotheses

1. Administration of oral L-arginine, but not D-arginine, to male Sprague Dawley rats will alter the expression/levels and or activity of enzymes, and metabolic products of several metabolic pathways using L-arginine as a substrate. There will be a decrease in the basal formation of methylglyoxal.
2. Administration of oral L-arginine, but not D-arginine, to Zucker Diabetic Fatty rats, will attenuate increased mean arterial pressure, endothelial dysfunction and increased methylglyoxal levels.
3. Administration of oral L-arginine, but not D-arginine, to Zucker Diabetic Fatty rats will improve glucose tolerance and insulin response in an oral glucose tolerance test.

1.10.2 Objectives

1. Determine the impact of orally administered L-and D-arginine at two different doses of 500 mg/kg/day and 1000 mg/kg/day for 4 weeks and 16 weeks, respectively, on arginine metabolic pathways and the glyoxalase-mediated methylglyoxal degradation pathway, in male Sprague Dawley rats (Phase I).
2. To determine if a dose of 1000 mg/kg/day of L-or D-arginine for 16 weeks has a greater impact on arginine levels, mean arterial pressure and plasma methylglyoxal levels than a 500 mg/kg/day dose for 4 weeks, in male Sprague Dawley rats (Phase I).
3. Determine the effect of a 1000 mg/kg/day dose of L-and D-arginine for 12 weeks on arginine levels, and the eNOS, arginase and glyoxalase metabolic pathways in a rat model with elevated methylglyoxal levels, *viz*. male Zucker Diabetic Fatty rats (Phase II).
4. To investigate if L-and D-arginine supplements are more effective in improving endothelial function, lowering mean arterial pressure and reducing basal methylglyoxal formation in a pathological model (Phase II) compared to a physiological model (Phase I).
5. To understand the effect of administering 1000 mg/kg/day for 12 weeks of oral L- and D-arginine on glucose tolerance and insulin response in an oral glucose tolerance test in Zucker Diabetic Fatty rats (Phase II).

CHAPTER 2. MATERIALS AND METHODS

2.1 Animals:

All animal protocols were approved by the University of Saskatchewan's Animal Research Ethics Board, following guidelines of the Canadian Council on Animal Care and ARRIVE guidelines (Kilkenny et al., 2013). The experiments were carried out in three different phases using separate animals as follows:

Phase Ia: **Sprague Dawley rats** treated with **500 mg/kg/d** of L- or D-Arg for **4 weeks**

Phase Ib: **Sprague Dawley rats** treated with **1000 mg/kg/d** of L- or D-Arg for **16 weeks**

Phase II: Zucker lean and Zucker Diabetic Fatty (ZDF) rats treated with **1000 mg/kg/d** of L- or D-Arg for **12 weeks**

2.1.1 Phase Ia: Arginine 500 mg/kg/d for 4 weeks in Sprague Dawley rats

Twenty five, 9-week-old, male Sprague Dawley (SD) rats were purchased from Charles River Laboratories (Montreal, QC, Canada) for use according to protocol #20160059. The age of 9 weeks was chosen because rats become young adults at 9 weeks of age (Sengupta, 2013). The animals were randomly assigned to one of three treatment groups following a week of acclimatization. The treatment groups included (1) Control: plain drinking water (n = 8), (2) L-arginine: 500 mg/kg/day *via* drinking water (Cat # A-5006, Sigma-Aldrich, Oakville, ON, Canada) (n = 9), (3) D-arginine: 500 mg/kg/day *via* drinking water (Cat # GM7267, Glentham Life Sciences Ltd., Corsham, UK) (n = 8). L- and D-Arg were dissolved as free bases and the pH of the final stock solution was adjusted to 7.4 using hydrochloric acid (Cat # 51006G500C / 351280-500, Fisher Scientific, Ottawa, ON, Canada). Rats were treated for a total period of 4 weeks. The dose of 500 mg/kg/day was chosen based on the dose commonly used in rats (Fu et al., 2005; Matsuoka et al., 1996; Vosatka et al., 1998) and humans (Ast et al., 2011; Clarkson et al., 1996). The time period of 4 weeks was chosen as minimum for a chronic treatment period, and to allow us a second phase study with a longer 16-week period of treatment.

2.1.2 Phase Ib: Arginine 1000 mg/kg/d for 16 weeks in Sprague Dawley rats

Eighteen, 9-week-old male Sprague Dawley (SD) rats were purchased from Charles River Laboratories (Montreal, QC, Canada) for use according to protocol #20160059. The animals were randomly assigned to one of three treatment groups following a week of acclimatization. The

treatment groups included (1) Control: plain drinking water (*n* = 4), (2) L-arginine: 1000 mg/kg/day *via* drinking water (*n* = 7), (3) D-arginine: 1000 mg/kg/day *via* drinking water (*n* = 7). Rats were treated for a total period of 16 weeks. Initially, a dose of 2000 mg/kg/day was chosen as it is on the higher end of the dose range used in rats (Fu et al., 2005; Matsuoka et al., 1996; Vosatka et al., 1998) and humans (Ast et al., 2011; Clarkson et al., 1996). However, this dose was not approved by the Research Ethics Board due to a concern that it may cause pancreatitis, thus a dose of 1000 mg/kg/day was used. The higher dose was chosen to due lack of altered arginine levels with the 500 mg/kg/day dose and to compare and contrast against the lower dose. Similarly, the longer time period of 16 weeks *vs* 4 weeks was chosen to observe any differences between a short- and long-term treatment period.

2.1.3 Phase II: Arginine 1000 mg/kg/d for 12 weeks in Zucker rats

Fifteen male, 5-week-old, Zucker Diabetic Fatty (ZDF) rats and five male, 5-week-old, Zucker lean rats were purchased from Charles River Laboratories (Montreal, QC, Canada) for use according to protocol #20160059. Following one week of acclimatization, the Zucker lean animals were assigned to the Zucker lean control (ZLC) group (*n* = 5). The ZDF rats were randomly assigned to one of three treatment groups: (1) Control Zucker Obese (ZOC): plain drinking water (*n* = 5), (2) Zucker Obese + L-arginine (ZOA): L-Arg 1000 mg/kg/day *via* drinking water (*n* = 5), (3) Zucker Obese + D-Arginine (ZOD): D-Arg 1000 mg/kg/day *via* drinking water (*n* = 5). Treatment was administered for a total of 12 weeks. Treatment was started at 6 weeks of age as this is approximately when Zucker Diabetic Fatty rats begin to develop characteristics of diabetes mellitus (Owens, 2006). The smaller sample size of 5 was chosen due to the high cost of these animals, but still provide enough power to the statistical data. The dose of 1000 mg/kg/day was chosen due to a lack of altered arginine levels in the plasma and organs, in the 500 mg/kg/day phase Ia study in SD rats. A treatment period of 12 weeks was chosen as sufficient to observe any attenuating effects of arginine on mean arterial pressure increase, glucose intolerance and MG levels increase.

Arginine supplements were delivered through the drinking water for two reasons. The first being that when humans take arginine supplements it is most commonly through the oral route of administration. Secondly, performing an oral gavage on these animals every day for such an extended period of time would undoubtedly be extremely stressful on the animals and in a past

study by our lab resulted in high mortality rates. Such stress could be a confounding factor on results of the study. Daily oral gavage for 12 to 16 weeks is also not ethically sound. Furthermore, our animal house required that a trained technician performed the oral gavage and they could not provide someone every day for such an extended period. Rats were individually housed for the entirety of the treatment period to allow for individual water intake to be recorded daily. Additionally, body weight was recorded every other day. Each rat's daily water intake and body weight were used to calculate the volume of L- or D-arginine solution (10 g/L or 20 g/L, adjusted to pH 7.4) that when diluted to 300 mL with water would deliver the full required daily dose and avoid individual dose variations resulting from different water intakes. Water with adjusted arginine doses was changed every other day.

2.2 Measurement of mean arterial pressure:

Following completion of the treatment period, a terminal experiment was performed as follows. The rat was placed in a metabolic cage overnight (12 -16 h with access to water only) prior to the experiment in order to fast the animal and collect urine. Rats were placed on a homeothermic blanket to maintain the core body temperature at 37° C and anesthetized with isoflurane (Forane, 2-4% in oxygen) and a cannula filled with heparinized saline (50 U/mL) was inserted into the right femoral artery and connected to a pressure transducer to record the mean arterial pressure (MAP) (Desai et al., 2006). Another cannula (i.d. 0.5, o.d. 1.0 mm) was inserted into the right femoral vein to inject drugs and collect blood samples. The mean arterial pressure (MAP) was recorded for 20 min using the Powerlab and Chart software (AD Instruments Inc., Colorado Springs, CO, USA). The heart rate values were calculated from the Chart software. MAP was recorded using an intra-arterial cannula rather than by radio telemetry as MAP was not a primary focus of the study and radio telemetry is highly invasive and can increase the chance of infection as well as cause stress to the animal when used chronically. However, we opted not to record chronically as we were more interested in the effect of arginine supplements on MAP after the full treatment had been completed. As we only needed to record acutely, it was more practical to use an intra-arterial cannula as insertion into a major artery such as the abdominal artery could be avoided. It was our goal to minimize any risk of excess bleeding as much as possible due to the frailty and difficulty of working with Zucker rats. Upon completion of the MAP recording a 1 mL blood sample was drawn into an EDTA tube (100 µL of 50 uM EDTA (Cat # EX039-1 EMD Chemicals, Darmstadt,

Germany) in 1.5 mL microcentrifuge tube (Fisher Scientific, Ottawa, ON, Canada)). The plasma was separated *via* centrifugation and stored at -80°C for later analysis of arginine levels, enzyme activity levels, arginine related metabolite levels and MG levels.

2.3 Endothelium-dependent and -independent hypotensive responses:

Following completion of the basal blood pressure recording endothelial function was assessed in Phase Ib and II animals by administering bolus i.v. doses (0.125, 0.25, 0.5, 1, 2 and 4 µg/kg body wt.) of the endothelium-dependent muscarinic receptor agonist acetylcholine (ACh, Cat # A6625, Sigma-Aldrich, Oakville, ON, Canada)) and the endothelium-independent NO donor sodium nitroprusside (SNP, Cat # S0501, Sigma-Aldrich, Oakville, ON, Canada) *via* the femoral vein. The hypotensive responses to these agonists were recorded *via* the femoral artery cannula. Endothelial function was of greater interest than overall MAP as past studies in our lab have shown that increased MG levels associated with chronic diseases such as diabetes can cause endothelial dysfunction *in vitro* (Dhar et al. 2010). Furthermore, L- and D-Arg have shown the ability to attenuate this dysfunction *in vitro*, primarily through a MG scavenging effect (Dhar, et al., 2010).

2.4 Oral Glucose Tolerance Test

Upon completion of the hypotensive responses, an oral glucose tolerance test was performed on phase II animals using a protocol commonly performed in our lab (Dhar et al., 2011). In brief, the already anesthetized and cannulated (as described above) animal was kept at an internal body temperature of 37° C. A 1 mL sample at 0 min was collected following which a dose of 1 g g/kg body weight of glucose was injected directly into the stomach. For this, a small incision was made on the abdominal skin and underlying muscles under the left lowest rib where the stomach lies beneath the diaphragm. The exposed stomach was gently lifted and we ensured that the needle was inside the stomach cavity. Blood samples (0.5 mL each) were collected *via* the femoral artery into and EDTA tubes (100 µL of 50 µM EDTA (Cat # EX039-1 EMD Chemicals, Darmstadt, Germany) in 1.5 mL microcentrifuge tube (Fisher Scientific, Ottawa, ON, Canada)) at 5, 10, 15, 30, 60, 90 and 120 min. The samples were kept on ice and centrifuged at 12,000rpm for 10 min. Plasma was collected and stored at -80° C for later analysis. Plasma glucose and insulin levels were analyzed using appropriate assay kits described below. Finally, the heart was removed in order to euthanize

the animal. Various organs/tissues were harvested, rinsed in 0.9% saline and immediately frozen in liquid nitrogen for storage at -80°C.

2.5 Plasma Glucose Assay

Plasma glucose was quantified using an assay kit (Cat # 10009582, Cayman Chemical Co., Ann Arbor, MI, USA) which converted glucose to hydrogen peroxide which was then catalyzed to produce a pink dye read at 514 nm. To begin the assay, plasma samples were diluted 1:5 in the provided assay buffer. Standards and samples were added to a 96-well plate in duplicate. Standards and samples were plated as follows: 85 µL of provided assay buffer + 15 µL of sample or standard were added to individual wells. The reaction was started by adding 100 µL of provided Enzyme Reaction Mix to each well. The plate was allowed to incubate for 10 min at 37° C after which it was read at 510 nm using the Multiskan Spectrum microplate reader (Thermo Labsystems, Waltham, MA, USA). The values were plotted as a graph in Graphpad PRISM, which was used to calculate the area under curve (AUC) of the 0, 5, 15, 30, 60 90 and 120 min plasma glucose values graph.

2.6 Plasma Insulin Assay

Insulin levels in the plasma were quantified using a sandwich immunoassay kit (Cat # 80-INSRT-E01, ALPCO, Salem, NH, USA). No treatment of the samples was required prior to starting the assay. A volume of 10 µL of each standard, control and sample was added to the provided anti-insulin plate followed by 75 µL of provided Working Strength Conjugate. The plate was then incubated for 2 h at room temperature on a microplate shaker. The contents of the plate were then emptied, and each well was filled with provided Working Strength Wash Buffer. This washing procedure was repeated a total of six times. After the last wash any residual wash buffer and bubbles were removed. Next 100 µL of provided TMB Substrate was added to each well and the plate was again incubated for 15 min at room temperature on a microplate shaker. Finally, 100 µL of provided Stop Solution was added to each well and the plate was gently agitated to mix. The plate was read at 450 nm using the Multiskan Spectrum microplate reader (Thermo Labsystems, Waltham, MA, USA). The values were plotted as a graph in Graphpad PRISM, which was used to calculate the area under curve (AUC) of the 0, 5, 15, 30, 60 90 and 120 min plasma insulin values graph. A ratio of AUC (Glucose) / AUC (insulin) was then calculated.

2.7 Western Blotting for Enzyme and Transporter Expression

Enzyme expression was measured using standard western blotting protocols performed on cell lysates. The organs/tissues were ground into a fine powder under liquid nitrogen using a mortar and pestle and homogenized in a homogenization buffer (1% protease inhibitor cocktail, Cat # P8340 Sigma-Aldrich, Oakville, ON, Canada; in 1M Tris – 0.5 M EDTA + 0.3 M sucrose buffer) using a polytron homogenizer. Homogenized samples were then centrifuged for 10 min at 14000 rpm and the supernatant was collected. The protein concentration of each sample was determined using the DC protein assay (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). Next, 4x Laemmli sample buffer (Cat # 161-0737, Bio-Rad Laboratories, Hercules, CA, USA) + 2.5% 2-mercaptoethanol (Cat # M6250, Sigma-Aldrich, Oakville, ON, Canada) was added to the sample. Finally, the samples were denatured at 95°C for 5 minutes.

The samples prepared as stated above were then loaded using 40-75ug of protein from each sample into 50 µL wells of a 4-20% acrylamide precast gels (Cat # 456-1094, Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). The Precision Plus Protein Standard Dual Color Ladder (Cat # 161-0374, Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) was loaded alongside the samples at a volume of 5 µL into the first well. Proteins were separated in an electrode running buffer (15 g/L Tris-Base, Cat # 161-0719; 72 g/L glycine, Cat # 161-0724 and 15 g/L sodium dodecylsulfate, Cat # 161-0302, all Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) using electrophoresis as follows: first 5 minutes at 50V followed by 85 minutes at 100V for a total run time of 90 min. After completion of electrophoresis, proteins were then wet transferred in a transfer buffer (pH adjusted to 9.2; 5.85 g/L Tris-Base, 2.93 g/l glycine, 0.373 g/L sodium dodecylsulfate and 20% methanol, Cat # A452-4, Fischer Scientific, Ottawa, ON, Canada) for 3h at 4°C to a 0.45 µm polyvinylidene difluoride (PVDF, Cat # 45004110, GE Healthcare Life Sciences, Mississauga, ON, Canada) membrane.

The membrane was then blocked for 1h at room temperature using either 5% skim milk powder or 5% bovine serum albumin (BSA, Cat # A7906, Sigma-Aldrich, Oakville, ON, Canada) for non phospho- and phosphoproteins, respectively. The blocked membranes were then incubated with the primary antibody overnight at 4°C. Dilutions used for each primary antibody were as follows: SLC7A1 (CAT-1, 1:1000, Cat # ABIN5965961, Antibodies-Online Inc., Atlanta, GA, USA); eNOS (1:1000, Cat # 611852, BD Transduction Laboratories, Mississauga, ON, Canada); arginase

I (Cat # ab91279), arginase II (1:1000, Cat # ab203071); arginine decarboxylase (ADC, 1:500 to 1:1000, Cat # ab157214), agmatinase (1:1000), guanidinoacetate methyltransferase (GATM, 1:250, Cat # ab87062), glyoxalase I (1:200, Cat # ab96032) and beta actin (1:8000, Cat # ab16039) (all from Abcam Inc, Toronto, ON, Canada). All primaries were diluted in tris buffered saline with Tween (TBST, pH adjusted to 7.5; 2.423 g/L Tris-base, 8.766 g/L NaCl, (Cat # S271-3); and 0.1% TWEEN, (Cat # BP337-100, both from Fischer Scientific, Ottawa, ON, Canada) with 1% BSA. After thoroughly washing with TBST (5 washes for 5 min each) and applying the 1:10000 horseradish-peroxidase secondary antibody (Cat # 170-6576: anti-mouse; Cat # 170-6515: anti-rabbit, both from Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) in TBST with 5% BSA for 1h at room temperature, the blot was again washed as stated above. Blots were then imaged using the G:BOX Chemi XX6 (Syngene, Frederick, MD, USA) and Clarity Western Enhanced Chemiluminescence Substrate (Cat # 170-5061, Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). Finally, the blots were analyzed *via* densitometry using the GeneTools software (Syngene, Frederick, MD, USA).

2.8 Arginine Assay

Arginine levels in the plasma and various organs were quantified using an assay kit (Cat # OKEH02603, Aviva Systems Biology, San Diego, CA, USA). To the best of our knowledge, the anti-arginine antibody used in the ELISA does not differentiate between L- and D-Arg. The finely ground organ was homogenized in 1x phosphate-buffered saline (PBS; pH adjusted to 7.4; 8 g/L NaCl, Cat # S271-3; 0.2 g/L KCl, Cat # BP366-1; 0.24 g/L KH_2PO_4, Cat # 450200, all three from Fischer Scientific, Ottawa, ON, Canada, and 1.44 g/L Na_2HPO_4, Cat # SX0710-1, Merck, Darmstadt, Germany), and centrifuged to collect the supernatant for use in the assay. Plasma samples did not require further preparation. The prepared samples were diluted to a final volume of 50 µL using provided diluent solution and pipetted into wells of the anti-arginine 96-well plate. Immediately after which 50 µL of provided 1x Arginine-Biotin complex was added to each well (excluding the blank). The plate was then sealed and incubated for 60 min at 37°C. Liquid in wells was then removed *via* aspiration. The plate was then washed three times as follows: 200 µL of provided 1x wash buffer was added to each well and allowed to incubate for 2 min after which the liquid was removed *via* aspiration. Next, 100 µL of provided 1x avidin-HRP conjugate was added to each well and the plate was incubated for 45 min at 37°C. Liquid was discarded using aspiration

and three washes described above were repeated. At this point, 90 µL of provided TMB substrate was added to each well and the plate was left to incubate in the dark at 37°C for 15 min. Finally, 50 µL of the provided stop solution was added to each well and the optical density was read within 5 min at 450 nm using the Multiskan Spectrum microplate reader (Thermo Labsystems, Waltham, MA, USA).

2.9 Nitric Oxide Synthase Activity Assay

Nitric oxide synthase activity was quantified in various organs/tissues using an assay kit (Cat # K205, Biovision Inc. Milpitas, CA, USA). The kit reagents include L-arginine as a substrate for NOS, and the necessary co-factors. The NO generated is metabolized to nitrate and nitrite, which are measured as NO_X after the nitrate reductase converts nitrate into nitrite (Griess reaction). The deep purple colored product is read at 540 nm. In order to assay NOS activity exogenous L-Arg is provided in the reaction mix as a substrate for NOS to convert over a set incubation time of 1 h. For this assay finely ground organ/tissue samples were homogenized on ice with 200 µL of provided cold NOS assay buffer with a protease inhibitor cocktail (Cat # P8340 Sigma-Aldrich, Oakville, ON, Canada). Organ/tissue homogenates were then centrifuged at 10,000 rpm, 4°C for 10 min and the supernatant was transferred to a cold microcentrifuge tube. Protein concentrations for each sample were then assayed using the DC protein system (Bio-Rad Laboratories, Oakville, ON). To begin the assay, 40 µL of organ/tissue homogenate was loaded into wells of a 96-well plate. Next, 40 µL of provided Reaction Mix was added to each sample well and allowed to incubate at 37°C for 1 h. Following the incubation, 95 µL of provided NOS Assay Buffer was added to each sample well followed by 5 µL of provided enhancer. The plate was gently agitated to mix and incubated at room temperature for 10 min. Finally, 50 µl of Griess Reagent 1 (sulfanilamide) and 50 µl of Griess Reagent 2 (N-1-napthylethylenediamine dihydrochloride) were added to each sample well and incubated for 10 min at room temperature. Absorbance was read at 540 nm using the Multiskan Spectrum microplate reader (Thermo Labsystems, Waltham, MA, USA).

2.10 Nitrates and nitrites Assay

Total nitrates and nitrites (NO_X = NO_3 + NO_2) were measured in the plasma and various organs/tissues as an indicator of NO production. Like the NOS activity assay described above, this

assay kit utilizes the Griess reaction to convert nitrate to nitrite which is then converted into a deep purple azo compound read at 540 nm (Cat # 780001, Cayman Chemicals, Ann Arbor, MI, USA). Plasma samples were ultra-filtered using 10 kDa molecular cut-off filters (Cat # MRCPRT010, Millipore Canada Ltd., Etobicoke, ON, Canada). Organ/tissue samples were finely ground and homogenized in a PBS buffer (pH 7.4). The supernatant was removed by centrifuging at 10,000 rpm for 20 min. The supernatant was then ultra-filtered in the same manner as the plasma samples. To begin the assay, 40 µL of sample plus 40 µL of provided assay buffer were added to the wells of a 96-well plate. Next 10 µL of the provided Enzyme Cofactor Mixture followed by 10 µL Nitrate Reductase Mixture was added to each sample well. The plate was then covered and incubated for 1 h at room temperature. Upon completion of the incubation time 50 µL of the provided Griess Reagent 1 (sulfanilamide) was added to each sample well immediately followed by 50 µL of the provided Griess Reagent 2 (N-1-napthylethylenediamine dihydrochloride). Color was allowed to develop for 10 min at room temperature after which the absorbance was read at 540 nm using the Multiskan Spectrum microplate reader (Thermo Labsystems, Waltham, MA, USA).

2.11 Arginase Activity Assay

Arginase is responsible for converting arginine into ornithine and urea. Arginase activity was measured in the plasma and various organs using a colorimetric assay kit (Cat # MAK112, Sigma-Aldrich, Oakville, ON, Canada). Plasma samples were filtered using a 10 kDa molecular weight cut-off filter (Cat # MRCPRT010, Millipore Canada Ltd., Etobicoke, ON, Canada) to remove urea as follows: from each plasma sample, 100 µL were loaded into the filter unit and diluted with 500 µL of water. The diluted plasma samples were then centrifuged at 14,000 rpm for 30 min. Once the plasma sample level was ~50 µL another 500 µL of water was added and centrifugation was repeated. Tissue/organ samples did not require further preparation. To begin the assay, 40 µL of each sample was added to wells of a 96-well plate. Next, 10 µL of provided 5x substrate buffer was added to each sample well and the plate was allowed to incubate for 2 h at 37°C. After incubation, 200 µL of the provided urea reagent was added to each sample well to stop the reaction. Again, 10 µL of the provided 5x substrate buffer was added to each sample well. The plate was gently agitated to mix and left to incubate for 60 min at room temperature. Finally, the absorbance was read at 430 nm using the Multiskan Spectrum microplate reader (Thermo Labsystems, Waltham, MA, USA).

2.12 Urea Assay

As a product of arginase metabolism, urea levels were measured in the plasma, urine and a variety of organs using an assay kit (Cat # ab83362, Abcam Inc, Toronto, ON, Canada). Organs samples were prepared by finely grinding organs and resuspending in the provided assay buffer. Samples were then homogenized on ice using a polytron homogenizer and centrifuged at 14000 rpm for 5 min. The supernatant was removed for use in the assay. Plasma and urine samples did not require any further preparation. Samples were loaded at a volume of 25 µL into wells of a 96-well plate followed by 25 µL of provided urea assay buffer and 50 µL of provided Reaction Mix. The plate was gently agitated to mix and incubated in the dark at 37°C. Upon completion of the incubation period the optical density was read at 570 nm using the Multiskan Spectrum microplate reader (Thermo Labsystems, Waltham, MA, USA).

2.13 Arginine Decarboxylase Assay

Arginine decarboxylase converts arginine into agmatine. The amount of this enzyme in the plasma and various organs was quantified using an assay kit based on a sandwich ELISA technology (Cat # abx251853, Abbexa Ltd., Cambridge Science Park, Cambridge, UK). In this assay the TMB substrate is catalyzed by HRP creating a blue color, once the stopping solution is added the color changes to yellow. The intensity of the yellow color read at 450 nm is proportional to the amount of ADC. Organ samples were finely ground and re-suspended in the same homogenization buffer described above in the western blot protocol. Once homogenized with a polytron homogenizer, samples were centrifuged for 10 min at 14000 rpm and the supernatant was collected for use in the assay. Plasma samples did not require any further preparation. To begin the assay, the provided ADC antibody coated 96-well plate was washed two times with the provided wash buffer. Next, 100 µL of each sample was added to the plate which was then incubated for 90 min at 37°C. After the incubation was completed, the liquid was removed, and the plate was washed two times with the provided wash buffer. A biotin conjugated antibody working solution was loaded at a volume of 100 µL to each well and the plate was sealed for incubation at 37°C for 60 min. The plate was then washed 3 times by filling each well completely with provided wash buffer and soaking for 1-2 min. Liquid was removed by tapping upside down onto a paper towel. Next, 100 µL of provided Streptavidin-HRP working solution was added to each well and the plate was

again incubated at 37°C for 30 min. Following incubation, the plate was washed 5 times as described above and 90 µL of TMB substrate was added to each well. The plate was then incubated in the dark for 15 min at 37°C after which 50 µL of the provided Stop solution was added to each well. Finally, the plate was gently agitated to mix and immediately read at 450 nm using the Multiskan Spectrum microplate reader (Thermo Labsystems, Waltham, MA, USA).

2.14 Total Polyamines Assay

Total polyamines include spermine, spermidine and putrescine. The levels of these polycations were measured in the plasma and various organs using a fluorometric assay kit (Cat # K475-100, BioVision Inc., Milpitas, CA, USA). Organ samples were finely ground, 10 mg of sample was added to 50 µl of provided cold assay buffer and homogenized on ice using a polytron homogenizer. The supernatant was collected by centrifuging samples at 10000 rpm for 5 min at 4°C. For every 100 µL of lysate, 2 µL of provided Sample Clean-up Mix was added to eliminate common metabolites that may interact with assay reagents, interfere with the assay or increase background. Samples were then incubated at room temperature for 30 min before being filtered *via* centrifugation at 10000 rpm for 10 min with a 10kD molecular weight cut off filter (Cat # MRCPRT010, Millipore Canada Ltd., Etobicoke, ON, Canada). Finally, the filtrate was collected for use in the assay. Plasma samples did not require any further preparation. The assay began with the loading of 10 µL of plasma samples plus 40 µL of provided Polyamine Assay Buffer to individual wells of a black 96-well plate. Organs were loaded into wells at a volume of 5 µL plus 45 µL of provided Polyamine Assay Buffer. Once the samples were loaded, 50 µL of Reaction Mix was added to each sample well and the plate was incubated for 30 min in the dark at 37°C. Hydrogen peroxide was the final product produced in the assay reaction which was read at excitation/emission wavelengths of 535/587 nm using the Fluoroskan Ascent microplate reader (Thermo Labsystems, Waltham, MA, USA).

2.15 Creatinine Assay

Creatinine is the final product of the arginine: glycine amidinotransferase pathway. Creatinine levels in the plasma, urine and various organs were measured using a colorimetric assay which uses a coupled enzyme reaction to produce a product that can be read at 570 nm and is proportional to the amount of creatinine in each sample. (Cat 3 MAK080, Sigma-Aldrich, Oakville, ON,

Canada). Organ samples were prepared by finely grinding and homogenizing on ice in provided cold creatinine assay buffer. Samples were then centrifuged at 13000 g for 10 min at 4 °C to collect supernatant. The supernatant was then filtered using a 10 kDa molecular weight cut off centrifugal filter (Cat # MRCPRT010, Millipore Canada Ltd., Etobicoke, ON, Canada) to removed excess protein which may have interfered with the assay. Plasma samples were also filtered to deproteinate. The samples were then loaded into wells of a 96-well plate as follows: plasma and organs – 40 µL of sample plus 10 µl of provided creatinine assay buffer, urine – 10 µL of sample plus 40 µL of provided creatinine assay buffer. Next, 50 µL of provided Reaction Mix was added to each sample well and the plate was gently agitated to mix. The plate was then incubated in the dark for 60 min at 37°C and absorbance was subsequently read at 570 nm using the Multiskan Spectrum microplate reader (Thermo Labsystems, Waltham, MA, USA).

2.16 Glyoxalase I Activity Assay

Glyoxalase I is the rate limiting enzyme of the glyoxalase system which detoxifies MG. This enzyme produces S-lactoylglutathione. Activity of glyoxalase I in various organs was quantified using an assay kit which monitored the change in absorbance at 240 nm (Cat # MAK114, Sigma Aldrich, Oakville, ON, Canada). Organ samples were prepared by homogenizing finely grounding the organ in the same homogenization buffer described in the western blotting protocol above. The homogenized samples were then centrifuged for 10 min at 14000 rpm and the supernatant was collected. Collected supernatants were divided into two microcentrifuge tubes (one for the sample reaction and one for the sample blank) with 40 µL of sample per tube. To the sample reaction tubes 160 µL of provided Master Reaction mix was added and allowed to incubate for 20 min at room temperature. Sample reaction tubes were then deproteinated by adding 70 µL of 4 M perchloric acid (PCA, Cat # 311413, Sigma Aldrich, Oakville, ON, Canada), vortexed to mix and incubated for 15 min on ice. Upon completion of incubation, sample reaction tubes were centrifuged at 14000 rpm for 5 min and 200 µL of supernatant was loaded into wells of a 96-well plate. Next, 70 µL of 4 M PCA was added to each sample blank tube. The tubes were then vortexed allowed to incubate for 15 min on ice and then centrifuged as described above. Next, 160 µL of provided Master Reaction Mix was added to each sample blank tube. The tubes were vortexed and incubated on ice for 15 min prior to centrifuging for 5 min at 14000 rpm. Finally, 200 µL of collected supernatant

from each sample blank tubes were added to the plate and absorbance was read at 240 nm using the Multiskan Spectrum microplate reader (Thermo Labsystems, Waltham, MA, USA).

2.17 Measurement of Methylglyoxal

Measurement of MG was performed *via* high performance liquid chromatography (HPLC) using a protocol standardized in our lab (Dhar et al., 2009). The organs frozen at -80 °C were finely ground under liquid nitrogen using a mortar and pestle. The powdered organs were then reconstituted in sodium phosphate monobasic buffer (50 mM, pH 7.4, Cat # SX0710-1, Merck, Darmstadt, Germany) with 50 µM EDTA (Cat # EX039-1 EMD Chemicals, Darmstadt, Germany). The samples were then vortexed and kept on ice during homogenization using a polytron homogenizer. During homogenization, the samples were sonicated for 30s pulses, 3 times. Homogenized samples were then centrifuged at 12000 rpm for 5 min and the supernatant was collected. The protein concentration of each sample was then determined using the DC protein assay (Bio-Rad Laboratories, Mississauga, ON, Canada).

The collected supernatant was then incubated with 0.45 N (final concentration) PCA and 10 mM (final concentration) of *o*-phenylenediamine (*o*-PD, Cat # P9029, Sigma Aldrich, Oakville, ON, Canada) for 24 h at room temperature in a box protected from light. PCA is added in order to precipitate proteins prior to the addition of *o*-PD as well as inhibit any metabolic reactions. The addition of *o*-PD is essential to act as a thermodynamic trap to derivatize free MG into stable 2-methylquinoxaline (2-MQ, Dhar et al., 2009). The following day, the incubated samples are centrifuged at 12000 rpm for 10 min. Next, 252 µL of the supernatant is collected into a separate microcentrifuge tube. The internal standard 5-methylquinoxaline (5-MQ) is added at a volume of 28 µL for a final concentration of 10 µM. The samples were then loaded (100 µL) in duplicates into HPLC vials ensuring no air bubbles were present. A Phenomenex kinetex C18 (5 µm, 100 Å) column (150 x 4.6 mm) with a mobile phase consisting of 20% acetonitrile (Cat # A998-4, Fischer Scientific, Ottawa, ON, Canada) and 8% sodium phosphate (pH 4.5, Cat # SX0710-1, Merck, Darmstadt, Germany) in distilled water was used to run samples through the column at a flow rate of 1 ml/min. Peaks for 2-MQ and the 5-MQ internal standard were detected at wavelength of 350 nm using a UV detector on a Hitachi D-7000 HPLC system (Hitachi Ltd., Mississauga, ON, Canada). Retention times for 2-MQ and 5-MQ were approximately 7 min and 14 min, respectively.

The ratio of the area under the curve for 2-MQ and 5-MQ peaks was used to calculate MG concentration from a 5 point standard curve of known 2-MQ concentrations (0.3 µM, 0.5 µM, 1 µM, 5 µM and 10 µM). Finally, the concentration of MG in each organ/tissue was standardized using the protein concentrations determined from each sample.

2.18 D-lactate Assay

D-lactate is the final product produced by MG metabolism *via* the glyoxalase system. Levels of D-lactate in the plasma, urine and various organs was quantified using an assay kit which oxidizes D-lactate *via* D-lactate hydrogenase to produce a coloured product which can be read at 450 nm (Cat # MAK058, Sigma-Aldrich, Oakville, ON, Canada). Organ samples were finely ground and homogenized in 100 µL of provided ice-cold assay buffer using a polytron homogenizer. The homogenized samples were then centrifuged at 10000 rpm for 10 min and the supernatant was collected for use in the assay. Plasma and urine samples did not require any further preparation. To begin the assay, 10 µL of each sample plus 40 µL of provided D-lactate assay buffer was added to wells of a 96-well plate. Next, 50 µL of the provided Reaction Mix was added to each sample well and the plate was agitated to mix. The plate was then incubated in the dark for 30 min at room temperature. Finally, the optical density was read at 450 nm using the Multiskan Spectrum microplate reader (Thermo Labsystems, Waltham, MA, USA).

2.19 Statistical Analysis

Results were statistically analyzed using One Way Analysis of Variance (ANOVA) followed by the Tukey's post hoc test to determine any significant differences between pairs of every group assayed. To analyze results of dose-response curves obtained with ACh and SNP we first calculated the area under curve (AUC) for each graph of each treatment group and then analyzed using One-way ANOVA and a post-hoc multi-comparisons test. Similarly, to analyze the results of OGTT for plasma glucose and insulin graphs we first calculated the AUC values for each graph of each treatment group and then analyzed using One-way ANOVA and a post-hoc multi-comparisons test. A *P*-value of less than 0.05 was considered significant. Data is represented as mean + standard error of mean (SEM).

CHAPTER 3. RESULTS

3.1 Oral arginine supplements (500 mg/kg/day) for 4 weeks did not result in mortality in Sprague-Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 4 weeks with 500 mg/kg/day of L-Arg or D-Arg did not result in mortality.

3.2 Oral arginine supplements (1000 mg/kg/day) for 16 weeks did not result in mortality in Sprague-Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 16 weeks with 1000 mg/kg/day of L-Arg or D-Arg did not result in mortality.

3.3 Oral arginine supplements (1000 mg/kg/day) for 12 weeks did not result in mortality in Zucker lean or Zucker Diabetic Fatty rats.

Treatment of 6-week-old male Zucker lean and Zucker Diabetic Fatty rats for 12 weeks with 1000 mg/kg/day of L-Arg or D-Arg did not result in mortality. One animal from the L-Arg group died during surgery as it bled out during the cannulation process. In the D-Arg group one animal was euthanized at the end of the treatment period as its hind limbs were blue and it was having trouble walking. We are unsure if this was due to the D-Arg supplements but as this was the only incident across all three studies we believe it was unrelated.

3.4 Oral arginine supplements (500 mg/kg/day) did not significantly alter daily water intake between treatment groups of Sprague Dawley rats after 4 weeks.

Treatment of 10-week-old male Sprague Dawley rats for 4 weeks with 500 mg/kg/day of L-Arg or D-Arg did not significantly alter daily water intake between individual rats or treatment groups (Fig. 3.1). The L-Arg group did have significantly higher water intake at the beginning of the treatment period (10 weeks of age) as compared to the control group but this difference was normalized at the end of the treatment period (14 weeks of age, Fig. 3.1).

3.5 Oral D-arginine supplements (1000 mg/kg/day) significantly decreased daily water intake of Sprague Dawley rats after 16 weeks.

Treatment of 10-week-old male Sprague Dawley rats for 16 weeks with 1000 mg/kg/day of L-Arg did not significantly alter daily water intake between individual rats or treatment groups (Fig. 3.2). Treatment with D-Arg did not significantly alter water intake between individual rats but those in the D-Arg group were consuming significantly less water at the end of the treatment period as compared to the L-Arg group (Fig. 3.2).

3.6 Oral arginine supplements (1000 mg/kg/day) did not significantly alter daily water intake between different treatment groups of Zucker rats after 12 weeks.

Treatment of 6-week-old Zucker Diabetic Fatty rats with 1000 mg/kg/day of L- or D-Arg did not significantly alter daily water intake at 18 weeks as compared to the Zucker obese controls (Fig. 3.3). Water intake was significantly increased at 18 weeks in the ZOC, ZOA and ZOD groups as compared to ZLC (Fig. 3.3). Daily water intake was significantly also increased in the ZOC, ZOA and ZOD groups at 18 weeks as compared to the intake at 6 weeks. Daily water intake was unchanged in the ZLC group at 18 weeks as compared to the ZLC at 6 weeks (Fig. 3.3).

3.7 Oral arginine supplements (500 mg/kg/day) did not significantly alter average body weight between treatment groups of Sprague Dawley rats after 4 weeks.

Treatment of 10-week-old male Sprague Dawley rats for 4 weeks with 500 mg/kg/day of L-Arg or D-Arg did not significantly alter average body weight between treatment groups (Fig. 3.4). The D-Arg group did have significantly higher average body weight at the beginning of the treatment period (10 weeks of age) as compared to the control and L-Arg group but this difference was normalized at the end of the treatment period (14 weeks of age, Fig. 3.4).

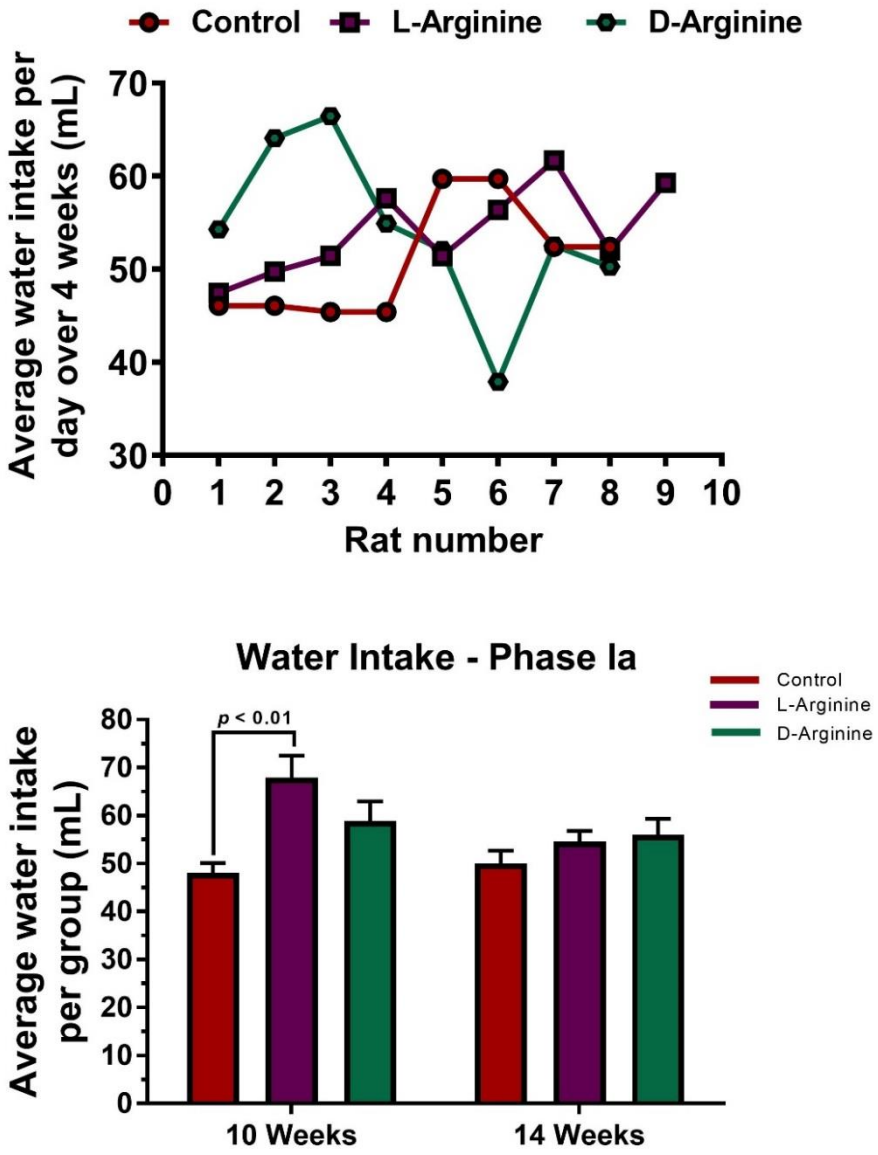


Figure 3.1. Effect of oral arginine (500 mg/kg/day) for 4 weeks on the daily water intake of Sprague Dawley rats.

10-week-old male Sprague Dawley rats were treated with L-arginine (L-Arg) or D-arginine (D-Arg) in drinking water (500 mg/kg/day) for 4 weeks. Daily water intake was measured by weighing the water bottles each day and calculating the difference in weight between the current day and the day before. (Control, n = 8; L-Arg, n = 9; D-Arg, n = 8).

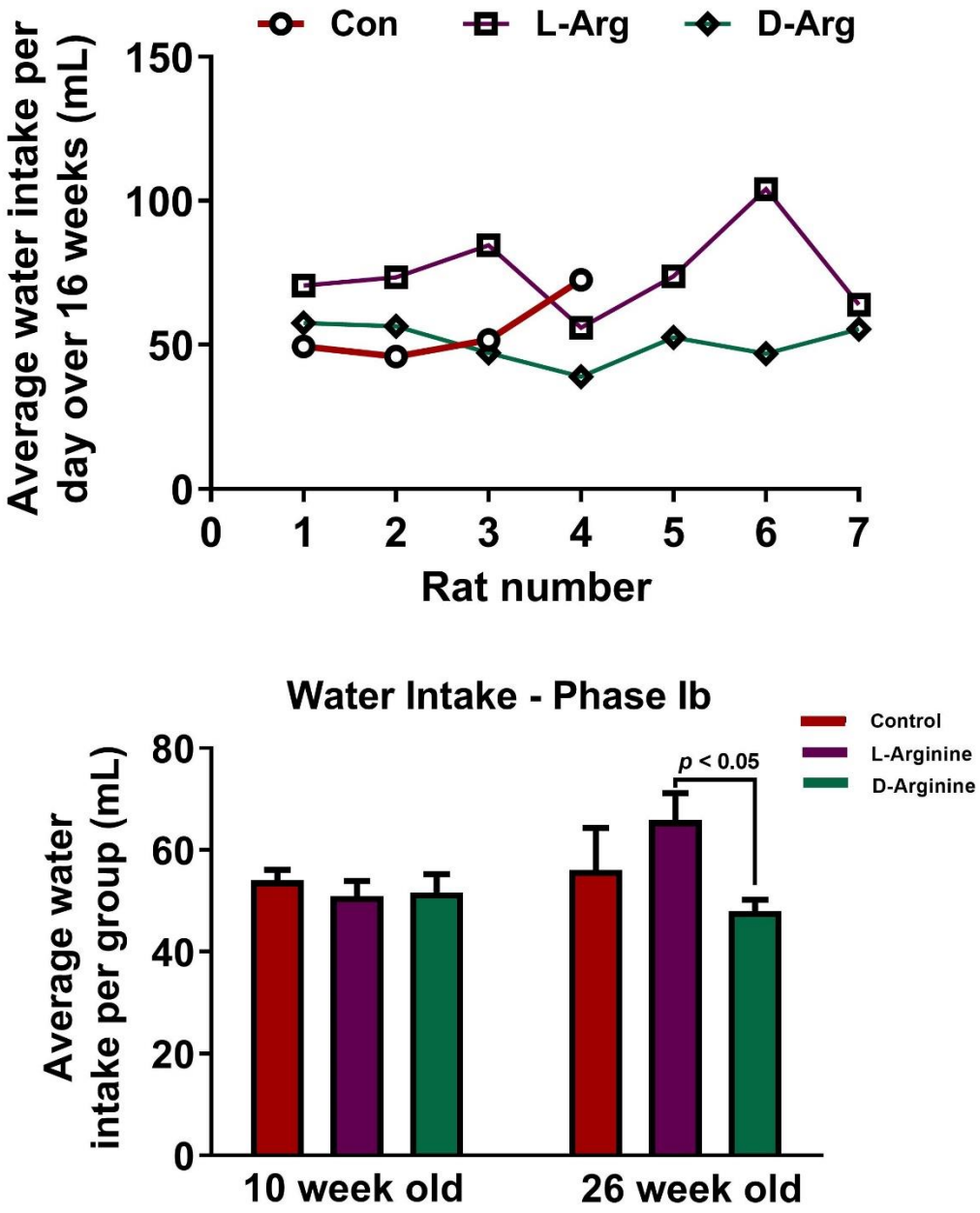


Figure 3.2. Effect of oral arginine (1000 mg/kg/day) for 16 weeks on the daily water intake of Sprague Dawley rats.

Ten-week-old male Sprague Dawley rats were treated with L-Arginine (L-Arg) or D-Arginine (D-Arg) in drinking water (1000 mg/kg/day) for 16 weeks. Daily water intake was measured by weighing the water bottles each day and calculating the difference in weight between the current day and the day before. (Control, n = 4; L-Arg, n = 7; D-Arg, n = 7).

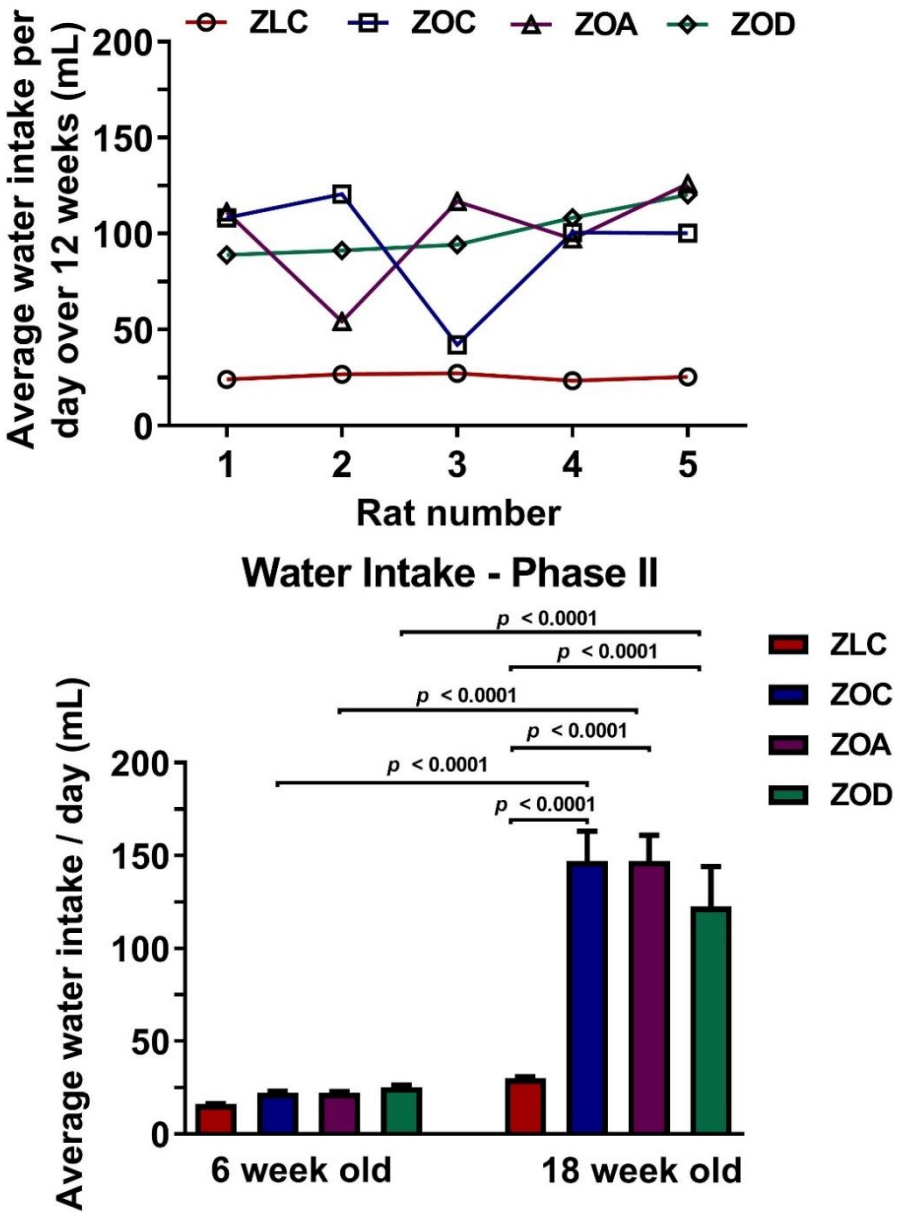


Figure 3.3. Effect of oral arginine (1000 mg/kg/day) for 12 weeks on the daily water intake of Zucker lean and Zucker Diabetic Fatty rats.

6-week-old male Zucker lean control (ZLC) and Zucker Diabetic Fatty control (ZOC) rats were given normal drinking water for 12 weeks. An additional group of Zucker Diabetic Fatty rats were treated with L-Arginine (ZOA) or D-Arginine (ZOD) in drinking water (1000 mg/kg/day) for 12 weeks. Daily water intake was measured by weighing the water bottles each day and calculating the difference in weight between the current day and the day before (n = 5).

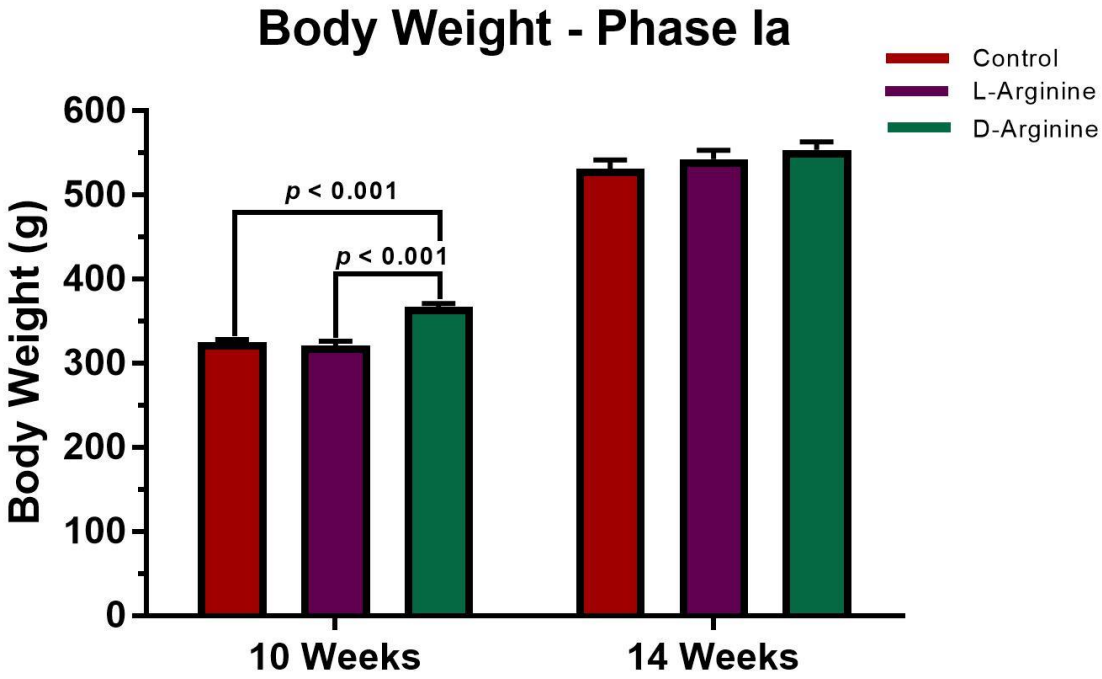


Figure 3.4. Effect of oral arginine (500 mg/kg/day) for 4 weeks on the average body weight of Sprague Dawley rats at 10 and 14 weeks.

10-week-old male Sprague Dawley rats were treated with L-Arginine (L-Arg) or D-Arginine (D-Arg) in drinking water (500 mg/kg/day) for 4 weeks. The average body weight of each group was calculated by weighing individual rats and calculating the group average (Control, n = 8; L-Arg, n = 9; D-Arg, n = 8).

3.8 Oral arginine supplements (1000 mg/kg/day) did not significantly alter average body weight between treatment groups of Sprague Dawley rats after 16 weeks.

Treatment of 10-week-old male Sprague Dawley rats for 16 weeks with 1000 mg/kg/day of L-Arg or D-Arg did not significantly alter average body weight compared to the control group or compared with each other (Fig. 3.5).

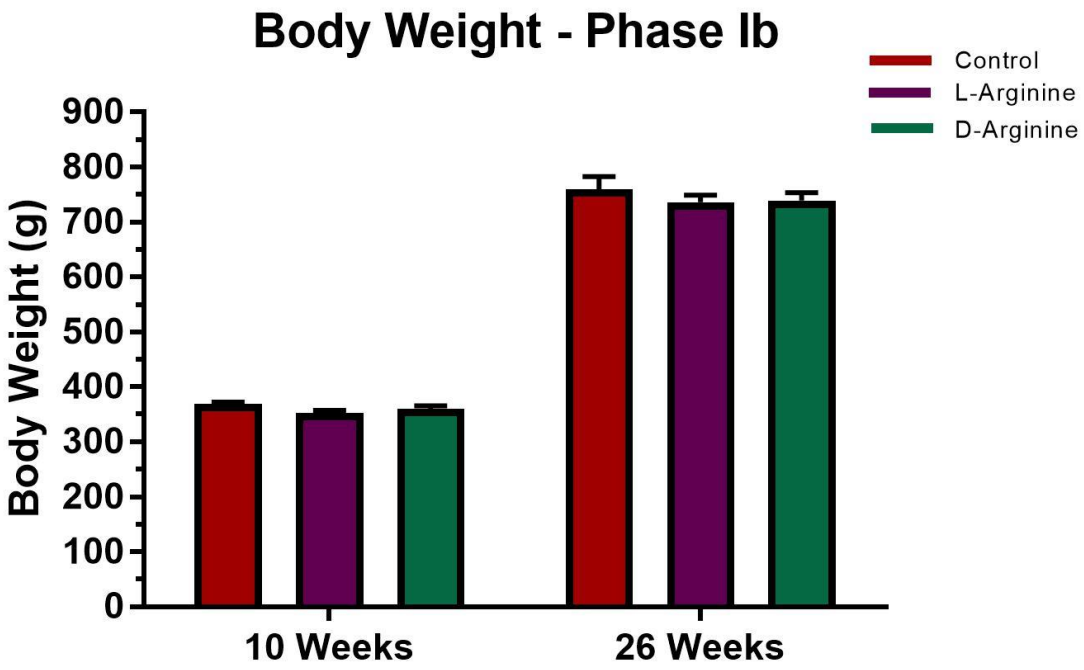


Figure 3.5. Effect of oral arginine (1000 mg/kg/day) for 16 weeks on the average body weight of Sprague Dawley rats at 10 and 26 weeks.

10-week-old male Sprague Dawley rats were treated with L-Arginine (L-Arg) or D-Arginine (D-Arg) in drinking water (1000 mg/kg/day) for 16 weeks. The average body weight of each group was calculated by weighing individual rats and calculating the group average (Control, n = 4; L-Arg, n = 7; D-Arg, n = 7).

3.9 Oral arginine supplements (1000 mg/kg/day) did not significantly alter average body weight between treatment groups of Zucker Lean or Zucker Diabetic Fatty rats after 12 weeks.

At six weeks of age Zucker Diabetic Fatty rats in the ZOC, ZOA and ZOD groups had significantly increased body weight as compared to the ZLC group consisting of Zucker lean rats (Fig. 3.6). After 12 weeks of treatment with either L-or D-Arginine supplements, Zucker Diabetic Fatty rats did not have significantly altered body weights as compared to the lean or obese controls (Fig. 3.6). Similarly, at 18 weeks, the Zucker obese controls did not have significantly altered weight as compared to the Zucker lean controls (Fig. 3.6).

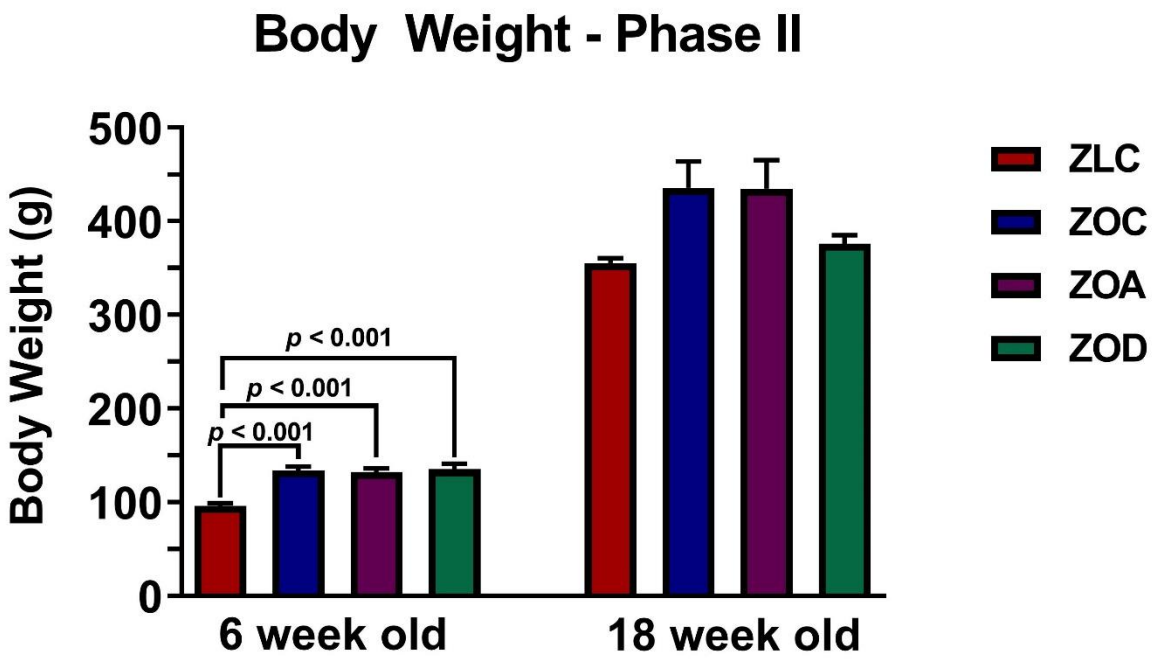


Figure 3.6. Effect of oral arginine (1000 mg/kg/day) for 12 weeks on the average body weight of Zucker lean and Zucker Diabetic Fatty rats at 5 and 17 weeks.

6-week-old male Zucker lean control (ZLC) and Zucker Diabetic Fatty control (ZOC) rats were given normal drinking water for 12 weeks. An additional group of Zucker Diabetic Fatty rats were treated with L-Arginine (ZOA) or D-Arginine (ZOD) in drinking water (1000 mg/kg/day) for 12 weeks. The average body weight of each group was calculated by weighing individual rats and calculating the group average (n = 5).

3.10 Oral arginine supplements (500 mg/kg/day) did not significantly alter arginine levels in the plasma, liver, kidney, ileum or brain of Sprague Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 4 weeks with 500 mg/kg/day of L-Arg or D-Arg did not significantly alter arginine levels in the plasma, liver, kidney, ileum or brain as compared to control group (Fig. 3.7).

3.11 Oral arginine supplements (1000 mg/kg/day) for 12 weeks did not significantly alter plasma, liver, ileum, kidney or urine levels of arginine in Zucker lean and Zucker Diabetic Fatty rats

Treatment of 6-week-old male Zucker lean and Zucker Diabetic Fatty rats for 12 weeks with 1000 mg/kg/day of L-Arg or D-Arg did not significantly alter arginine levels in the plasma, liver, kidney, ileum or brain as compared to control group (Fig. 3.8).

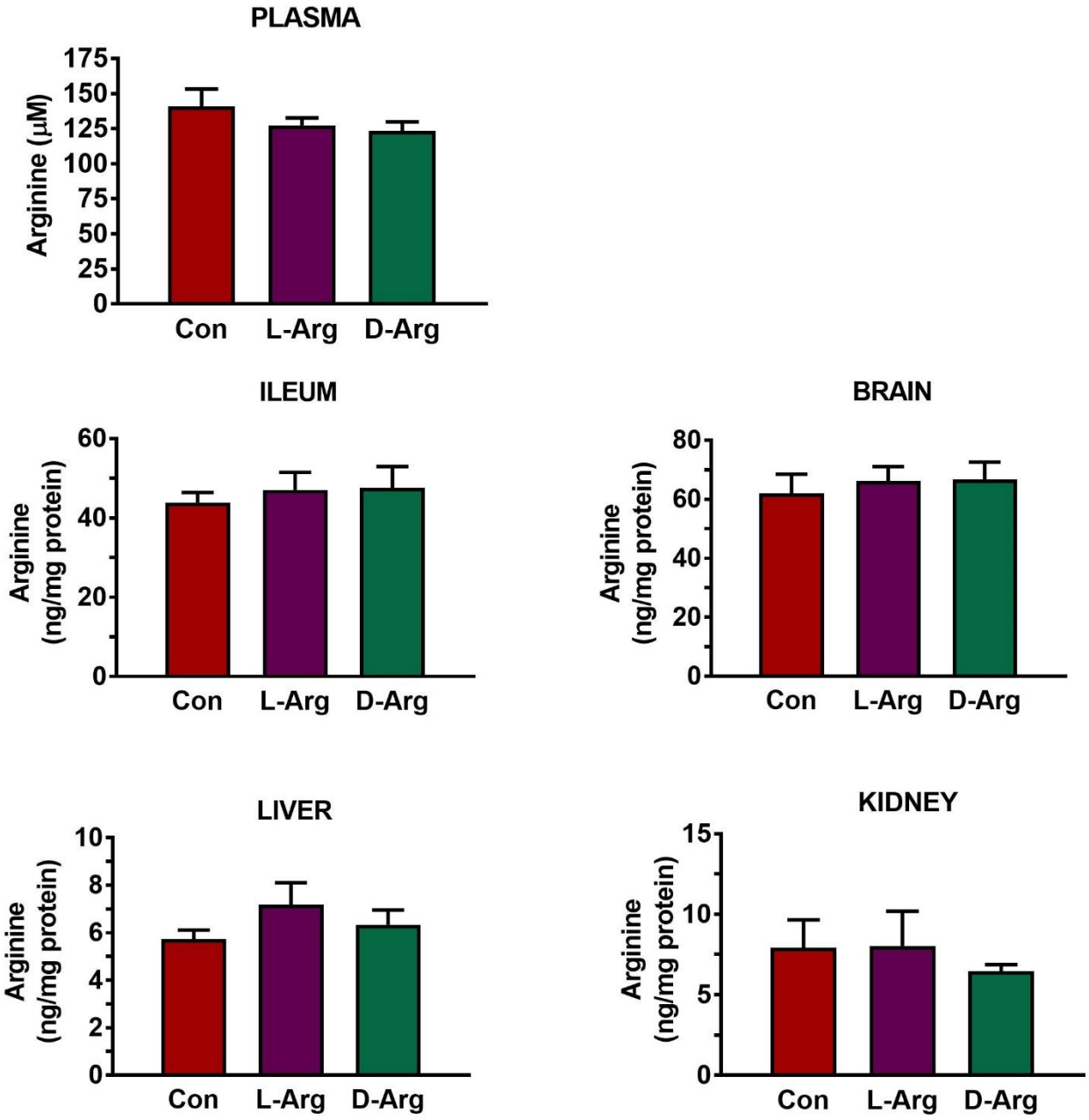


Figure 3.7. Effect of oral arginine (500 mg/kg/day) for 4 weeks on plasma and organs levels of arginine in Sprague Dawley rats.

10-week-old male Sprague Dawley rats were treated with L-Arginine (L-Arg) or D-Arginine (D-Arg) in drinking water (500 mg/kg/day) for 4 weeks. An assay kit was used to measure arginine levels. (Control, *n* = 4-6; L-Arg, *n* = 5-7*;* D-Arg, *n* = 5-7).

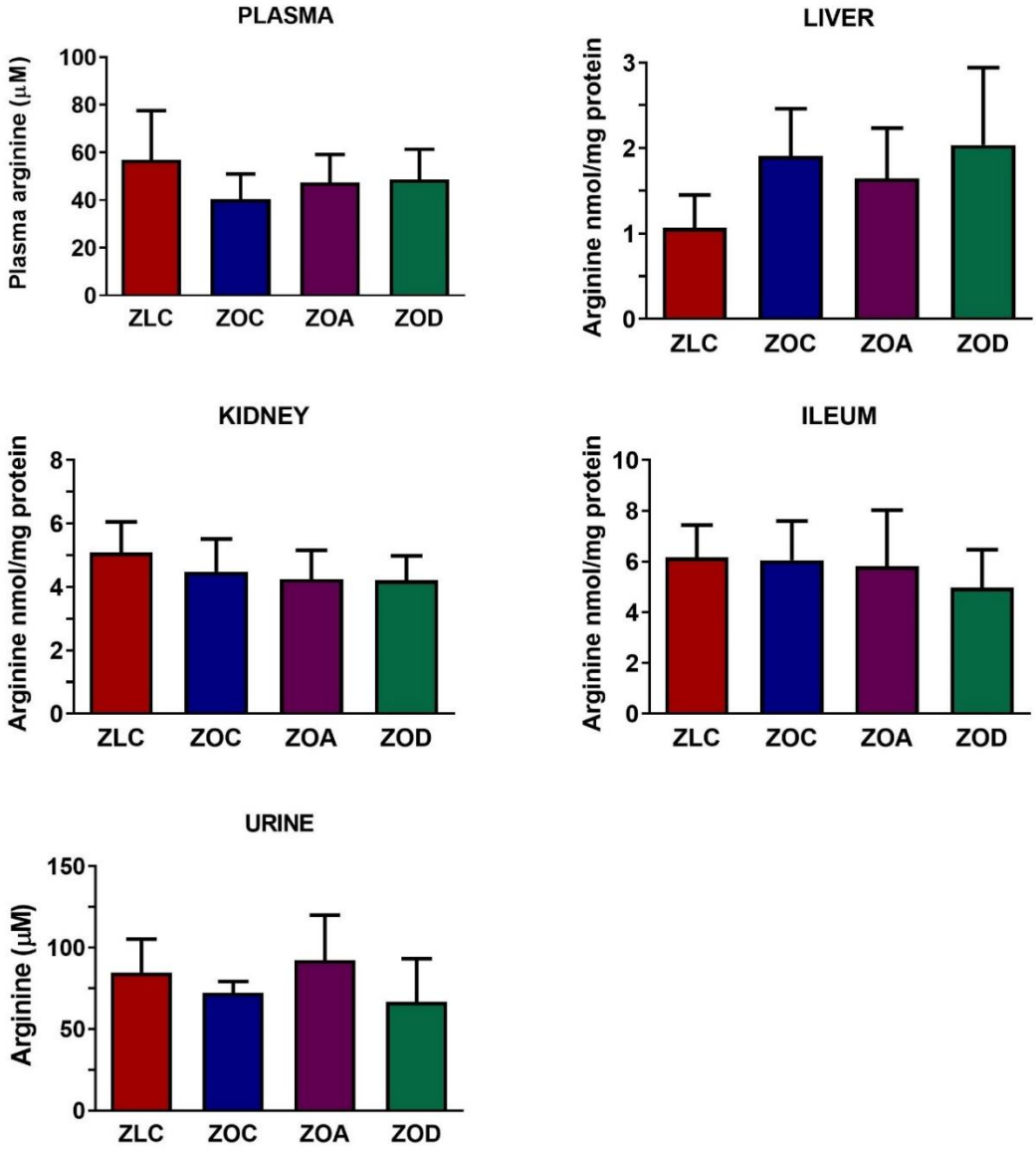


Figure 3.8. Effect of oral arginine (1000 mg/kg/day) for 12 weeks on plasma and organ levels of arginine in Zucker lean and Zucker Diabetic Fatty rats.

6-week-old male Zucker lean control (ZLC) and Zucker Diabetic Fatty control (ZOC) rats were given normal drinking water for 12 weeks. An additional group of Zucker Diabetic Fatty rats were treated with L-Arginine (ZOA) or D-Arginine (ZOD) in drinking water (1000mg/kg/day) for 12 weeks. An assay kit was used to measure arginine levels. (ZLC, *n* = 5; ZOC, *n* = 4-5; ZOA, *n* = 5; ZOD, *n* = 4-5).

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3.12 Oral L-Arginine supplements (500 mg/kg/day) significantly decreased expression of cationic amino transporter 1 (CAT-1) in the liver and ileum of Sprague Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 4 weeks with 500 mg/kg/day of L-Arg resulted in significant decreases in CAT-1 expression in the liver and ileum but not the aorta as compared to control (Fig 3.9). Treatment with 500 mg/kg/day of D-Arg for 4 weeks to 10-week-old male Sprague Dawley rats had no significant effect on CAT-1 expression in the liver, ileum or aorta (Fig. 3.9).

3.13 Oral arginine supplements (1000 mg/kg/day) significantly decreased expression of cationic amino transporter 1 (CAT-1) in the liver, ileum and aorta of Zucker Diabetic Fatty rats.

6-week-old Zucker Diabetic Fatty rats given normal drinking water for 12 weeks had significantly reduced expression of CAT-1 in the ileum and aorta but not the liver as compared to the Zucker lean controls (Fig. 3.10). Treatment of 6-week-old Zucker Diabetic Fatty rats with 1000 mg/kg/day of L-Arg or D-Arg for 12 weeks also significantly reduced expression of CAT-1 in the liver, ileum and aorta as compared to Zucker lean controls (Fig. 3.10).

3.14 Oral L- and D-Arginine supplements (500 mg/kg/day) did not significantly alter mean arterial pressure or heart rate in Sprague Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 4 weeks with 500 mg/kg/day of L-Arg or D-Arg did not significantly alter MAP (Fig. 3.11A) or heart rate (Fig. 3.11B) as compared to control. We were not able to measure systolic and diastolic pressures, which might have provided information on the effect of L-arginine on vascular smooth muscle tone.

3.15 Oral L- and D-Arginine supplements (1000 mg/kg/day) did not significantly alter mean arterial pressure or heart rate in Sprague Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 16 weeks with 1000 mg/kg/day of L-Arg or D-Arg did not significantly alter MAP (Fig. 3.12A) or heart rate (Fig. 3.12B) as compared to control.

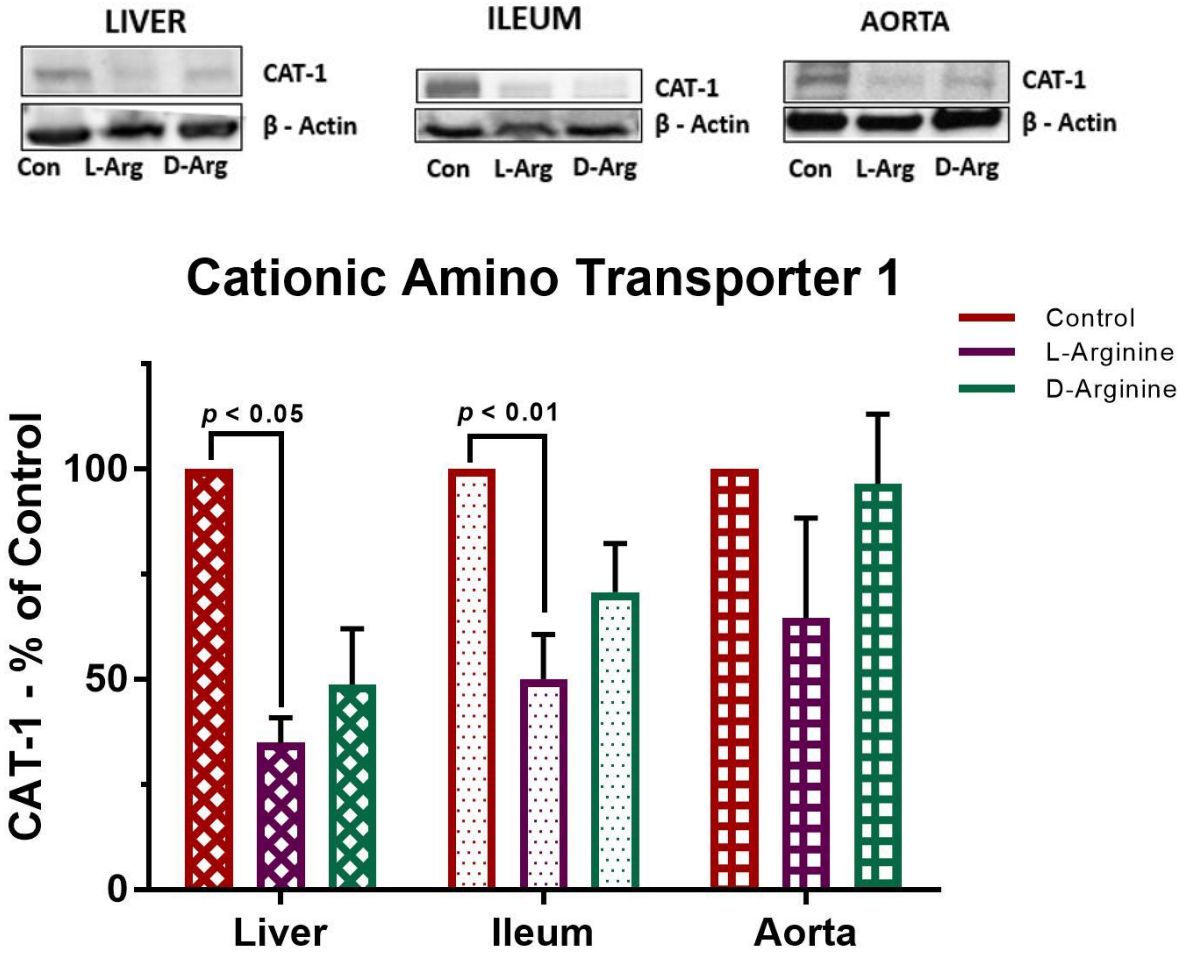

Figure 3.9. Effect of oral arginine (500 mg/kg/day) for 4 weeks on the expression of cationic transporter-1 (CAT-1, an arginine transporter) in the liver, ileum and aorta.

10-week-old male Sprague Dawley rats were treated with L-Arginine (L-Arg) or D-Arginine (D-Arg) in drinking water (500 mg/kg/day) for 4 weeks. Western blotting was used to determine expression of cationic amino acid transporter. The blots were quantified using the image analysis program GeneTools from Syngene. (Liver, n = 3-4; Ileum, n = 7-9; Aorta, n = 4).

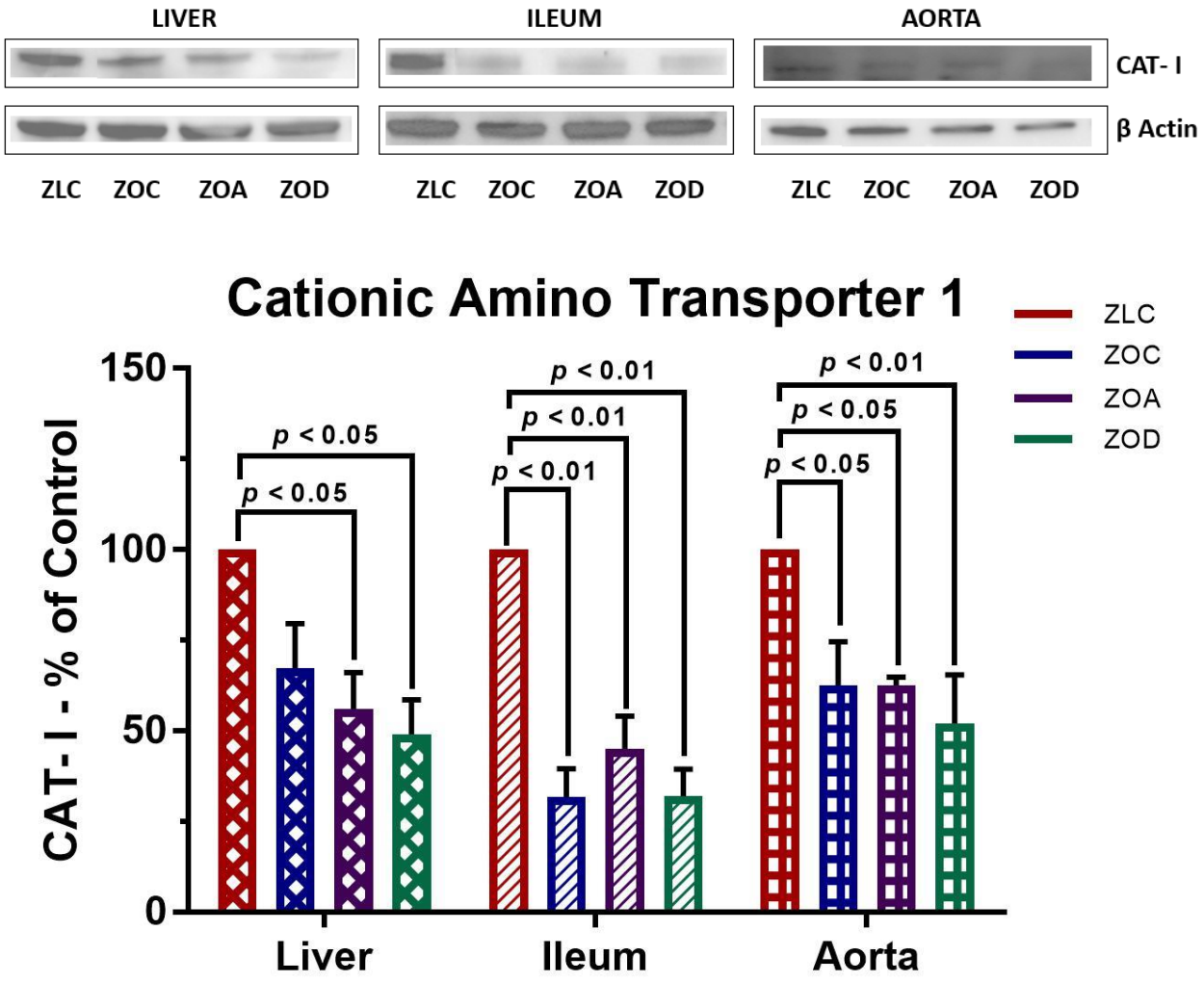

Figure 3.10. Effect of oral arginine (1000 mg/kg/day) for 12 weeks on the expression of cationic transporter-1 (CAT-1, an arginine transporter) in the liver, ileum and aorta of Zucker lean and Zucker Diabetic Fatty rats.

6-week-old male Zucker lean control (ZLC) and Zucker Diabetic Fatty control (ZOC) rats were given normal drinking water for 12 weeks. An additional group of Zucker Diabetic Fatty rats were treated with L-Arginine (ZOA) or D-Arginine (ZOD) in drinking water (1000 mg/kg/day) for 12 weeks. Western blotting was used to determine expression of cationic amino acid transporter. The blots were quantified using the image analysis program GeneTools from Syngene. (Liver, n = 3-4; Ileum, n = 3-6; Aorta, n = 3-4).

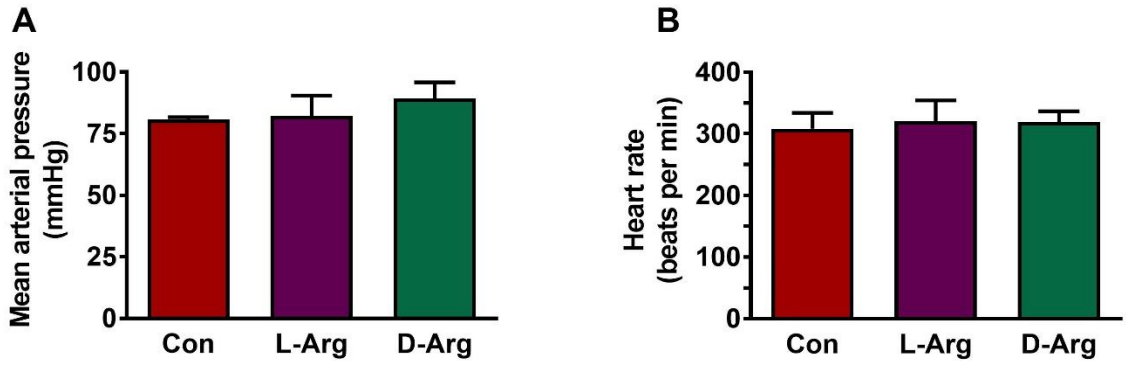

Figure 3.11. Effect of oral arginine (500 mg/kg/d) for 4 weeks on mean arterial pressure and heart rate in Sprague Dawley rats.

10-week-old male Sprague Dawley rats were treated with L-Arginine (L-Arg) or D-Arginine (D-Arg) in drinking water (500 mg/kg/day) for 4 weeks. Mean arterial pressure (A) and heart rate (B) were measured with a femoral artery catheter of anesthetized rats using PowerLab and Chart. (Control, *n* = 4; L-Arg, *n* = 5*;* D-Arg, *n* = 4).

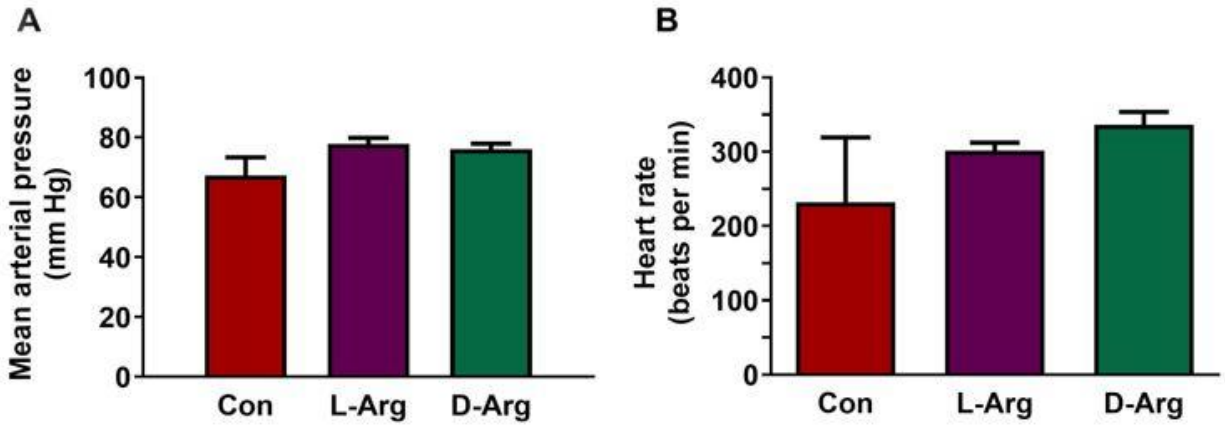

Figure 3.12. Effect of oral arginine (1000 mg/kg/d) for 16 weeks on mean arterial pressure and heart rate in Sprague Dawley rats.

10-week-old male Sprague Dawley rats were treated with L-Arginine (L-Arg) or D-Arginine (D-Arg) in drinking water (1000 mg/kg/day) for 16 weeks. Mean arterial pressure and heart rate were measured with a femoral artery catheter of anesthetized rats using PowerLab and Chart software. (Control, *n* = 4; L-Arg, *n* = 7*;* D-Arg, *n* = 7).

3.16 Oral L-Arginine supplements (1000 mg/kg/day) significantly reduced mean arterial pressure of Zucker Diabetic Fatty rats without affecting heart rate.

6-week-old Zucker Diabetic Fatty rats given normal drinking water for 12 weeks had significantly increased MAP as compared to the Zucker Lean controls (Fig. 3.13A). Treatment of 6-week-old male Zucker Diabetic Fatty rats for 12 weeks with 1000 mg/kg/day of L-Arg significantly reduced MAP as compared to the Zucker Diabetic Fatty rat controls (Fig. 3.13A). Treatment for the same time period with 1000 mg/kg/day of D-Arg did not significantly affect MAP (Fig. 3.13A). Neither L- or D-Arg had an effect on heart rate (Fig. 3.13B).

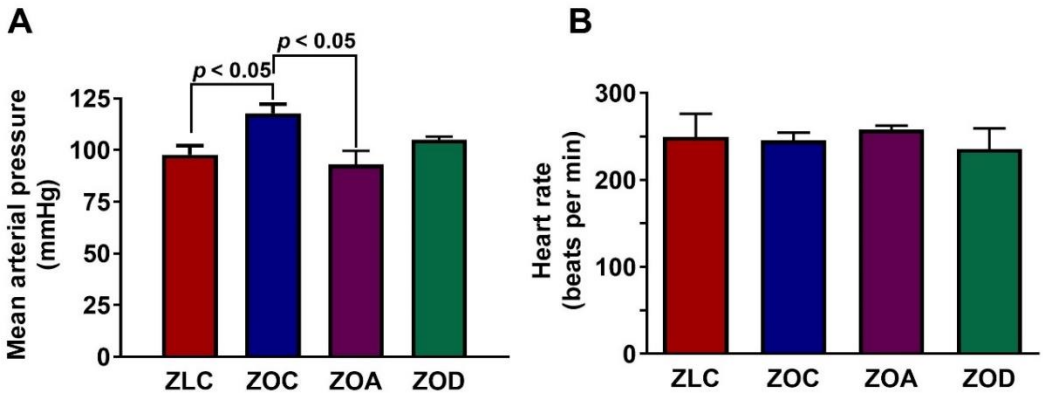

Figure 3.13. Effect of arginine (1000 mg/kg/d) for 12 weeks on mean arterial pressure in Zucker lean and Zucker Diabetic Fatty rats.

6-week-old male Zucker lean control (ZLC) and Zucker Diabetic Fatty control (ZOC) rats were given normal drinking water for 12 weeks. An additional group of Zucker Diabetic Fatty rats were treated with L-Arginine (ZOA) or D-Arginine (ZOD) in drinking water (1000 mg/kg/day) for 12 weeks. Mean arterial pressure (A) and heart rate (B) were measured with a femoral artery catheter of anesthetized rats using PowerLab and Chart software. (ZLC, n = 5; ZOC, n = 4; ZOA, n = 4; ZOD, n = 4).

3.17 Oral arginine supplements (1000 mg/kg/day) did not significantly alter endothelial-dependent vasodilation in Sprague Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 16 weeks with 1000 mg/kg/day of L- or D-Arg did not significantly alter endothelium-dependent responses to ACh (Fig. 3.14A, C) or endothelium-independent vasodilation to SNP (Fig. 3.14B, D). L-Arg does slightly enhance the

ability to vasodilate but not to a level that is statistically significant (Fig. 3.14). Due to bleeding we could not get hypotensive responses to all control rats. We accept the fact that this could increase the chances of a type 2 statistical error.

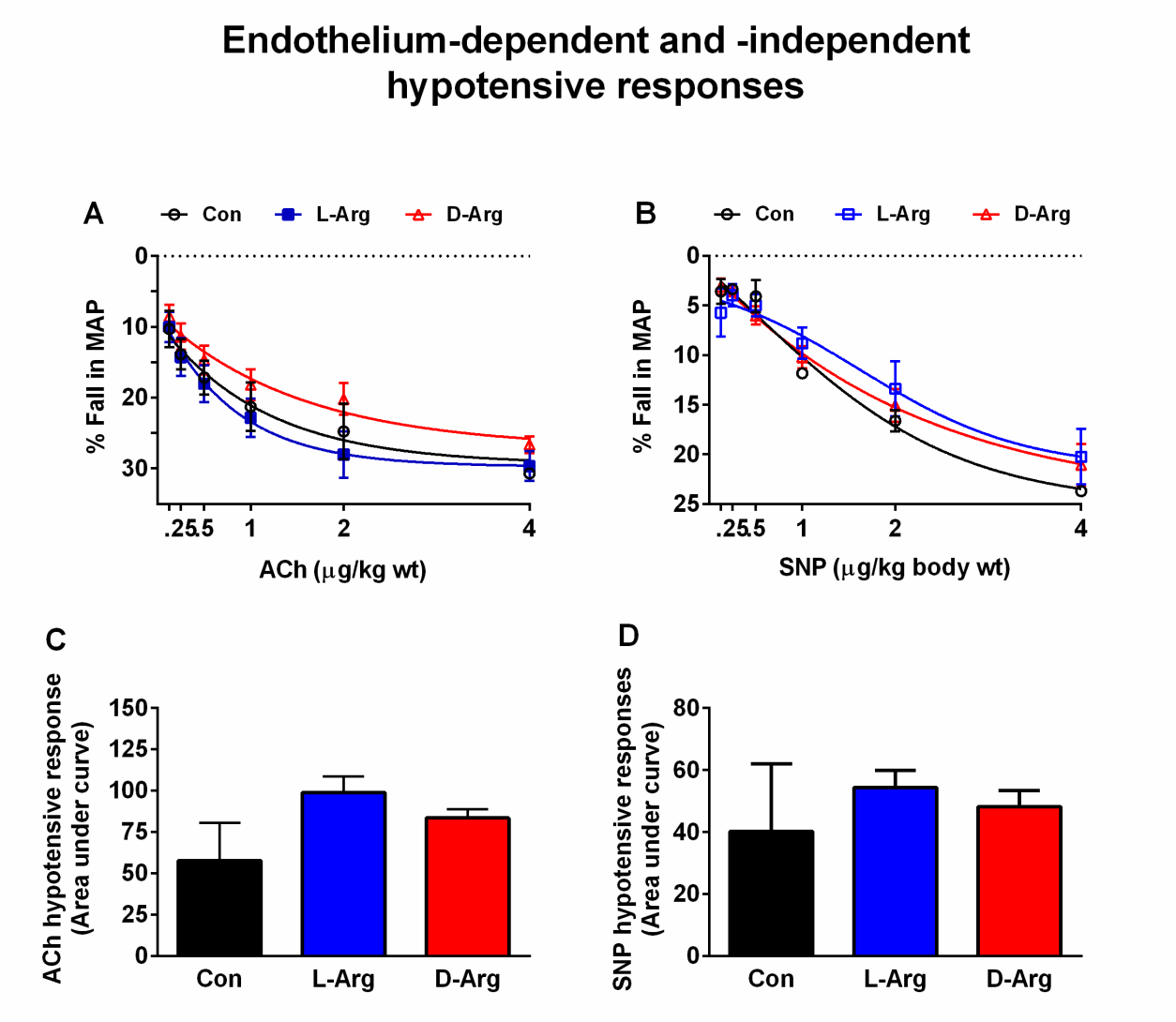

Figure 3.14. Effect of oral arginine (1000 mg/kg/d) for 16 weeks on endothelium-dependent and endothelium-independent vasodilation in Sprague Dawley rats.

10-week-old male Sprague Dawley rats were treated with L-Arginine (L-Arg) or D-Arginine (D-Arg) in drinking water (1000 mg/kg/day) for 16 weeks. Dose related hypotensive responses to acetylcholine (ACh) (A) and sodium nitroprusside (SNP) (B) bolus administration *via* the femoral artery were recorded from a femoral artery cannula in anesthetized rats using PowerLab and Chart software. Area under curve (AUC) values were then calculated (C, D) (Control, n = 3; L-Arg, n = 7; D-Arg, n = 7).

3.18 Oral L-Arginine supplements (1000 mg/kg/day) did not significantly improve endothelial function of Zucker Diabetic Fatty rats.

6-week-old Zucker Diabetic Fatty rats given normal drinking water for 12 weeks exhibited a trend toward diminished endothelial-dependent relaxation as compared to the Zucker Lean controls (Fig. 3.15). Treatment of 6-week-old male Zucker Diabetic Fatty rats for 12 weeks with 1000 mg/kg/day of L-Arg resulted in a trend towards an enhanced endothelial dependent relaxation (Fig. 3.15). Treatment for the same time period with 1000 mg/kg/day of D-Arg resulted in a trend similar to the obese control group (Fig. 3.15). Endothelium-independent vasodilation was also not significantly altered by either isomer (data not shown).

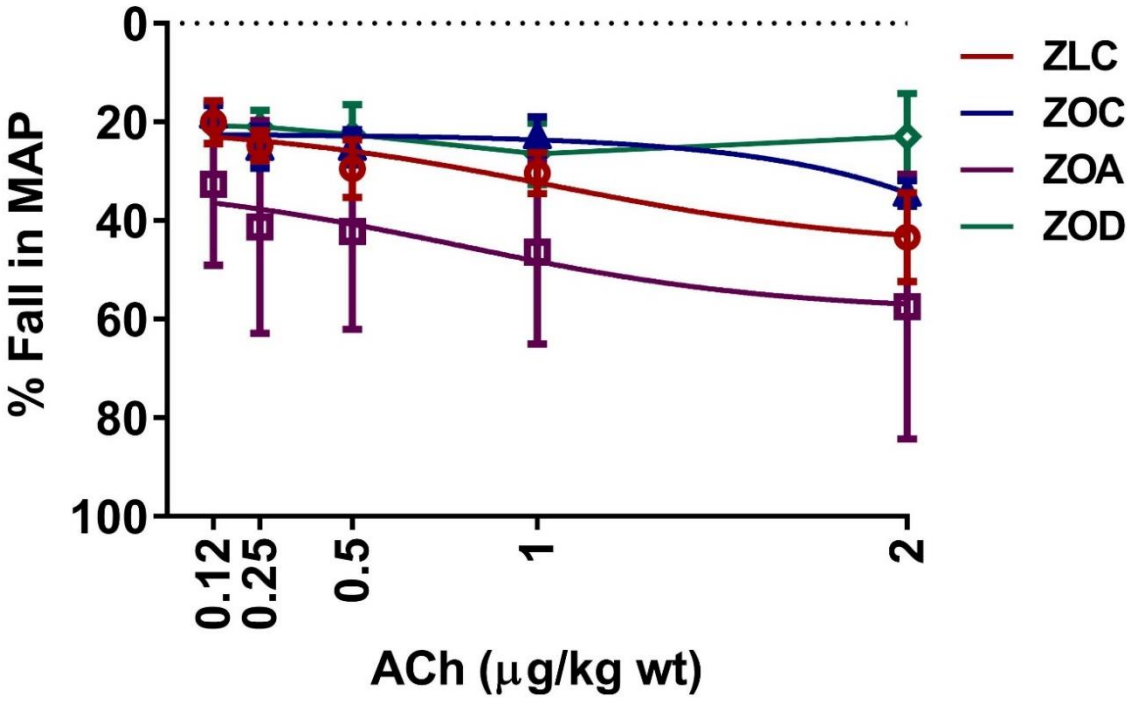

Figure 3.15. Effect of arginine (1000 mg/kg/d) for 12 weeks on endothelium-dependent vasodilation in Zucker lean and Zucker Diabetic Fatty rats.

6-week-old male Zucker lean control (ZLC) and Zucker Diabetic Fatty control (ZOC) rats were given normal drinking water for 12 weeks. An additional group of Zucker Diabetic Fatty rats were treated with L-Arginine (ZOA) or D-Arginine (ZOD) in drinking water (1000 mg/kg/day) for 12 weeks. Dose related hypotensive responses to acetylcholine bolus administration *via* the femoral artery were recorded from a femoral artery cannula in anesthetized rats using PowerLab and Chart software (ZLC, n = 5; ZOC, n = 4; ZOA, n = 2; ZOD, n = 4).

3.19 Oral arginine supplements (500 mg/kg/day) significantly increased expression of endothelial nitric oxide synthase (eNOS) in the aorta and kidney but not the brain of Sprague Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 4 weeks with 500 mg/kg/day of L-Arg significantly increased eNOS expression in the aorta and kidney (Fig. 3.16) compared to control. Treatment with 500 mg/kg/day of D-Arg for 4 weeks significantly increased eNOS in the kidney but not the aorta as compared to control. Brain eNOS expression was not significantly affected by either L- or D-Arg (Fig. 3.16).

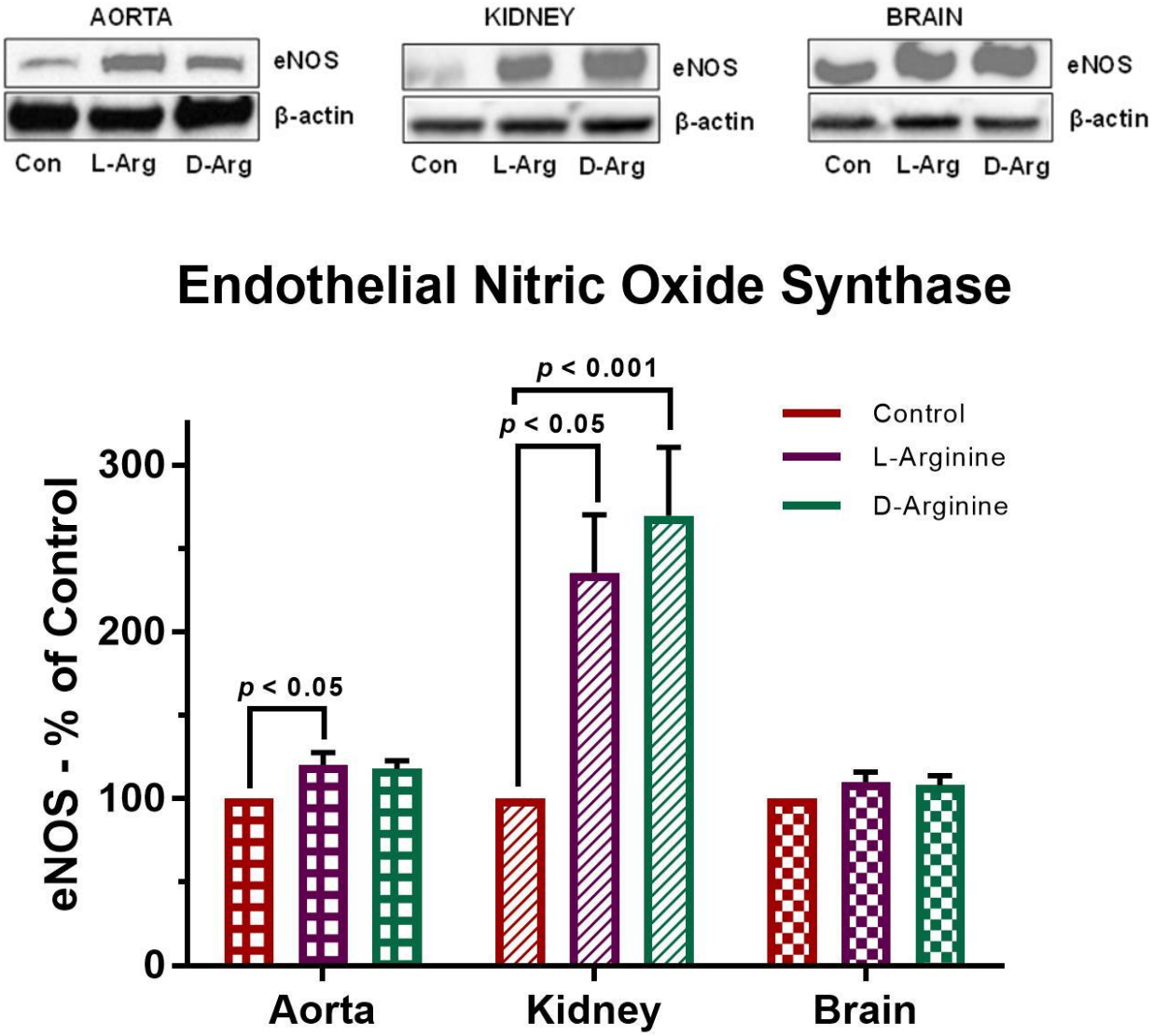

Figure 3.16. Effect of oral arginine (500 mg/kg/d) for 4 weeks on the expression of endothelial nitric oxide synthase in the aorta, kidney and brain of Sprague Dawley rats.

10-week-old male Sprague Dawley rats were treated with L-Arginine (L-Arg) or D-Arginine (D-Arg) in drinking water (500 mg/kg/day) for 4 weeks. Western blotting was used to quantify eNOS expression in the aorta, kidney and brain. The blots were quantified using the image analysis program GeneTools from Syngene (Aorta, n = 6-8; Kidney, n = 10-12; Brain, n = 15. "n" values represent the number of blots and not animals. n (no of animals) = 3 for each treatment group.

3.20 Oral arginine supplements (1000 mg/kg/d) significantly increased the expression of endothelial nitric oxide synthase in the aorta and kidney of Zucker lean and Zucker Diabetic Fatty rats.

6-week-old male Zucker Diabetic Fatty rat controls given normal drinking water for 12 weeks had significantly increased eNOS expression in the aorta but not the kidney as compared to Zucker lean controls (Fig. 3.17). Treatment of 1000 mg/kg/day with L-Arg for 12 weeks significantly increased eNOS expression in the aorta as compared to Zucker lean controls and in the kidney as compared to Zucker lean controls, Zucker Diabetic Fatty rat controls and the D-Arg treatment group (Fig. 3.17). Treatment with D-Arg significantly increased expression of eNOS in the aorta of Zucker Diabetic Fatty rats as compared to Zucker lean controls with no effect on expression in the kidney (Fig. 3.17).

3.21 Oral arginine supplements (500 mg/kg/day) significantly increased nitric oxide synthase (NOS) activity in the lungs of Sprague Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 4 weeks with 500 mg/kg/day of D-Arg significantly increased NOS activity in the lungs (Fig. 3.18) compared to control. Treatment with L-Arg did not significantly alter activity of NOS in the lungs (Fig. 3.18). Treatment with L-Arg or D-Arg did not significantly change NOS activity in the aorta, heart, kidney, ileum, liver, brain or skeletal muscle as compared to control (Fig. 3.18).

3.22 Oral arginine supplements (1000 mg/kg/day) did not significantly alter nitric oxide synthase activity in different organs/tissues of Zucker Diabetic Fatty rats.

Treatment of 6-week-old male Zucker Diabetic Fatty rats with 1000 mg/kg/day of oral L- or D-Arg for 12 weeks did not significantly alter nitric oxide synthase activity in the aorta, liver, kidney, ileum, heart, brain or lungs (Fig. 3.19).

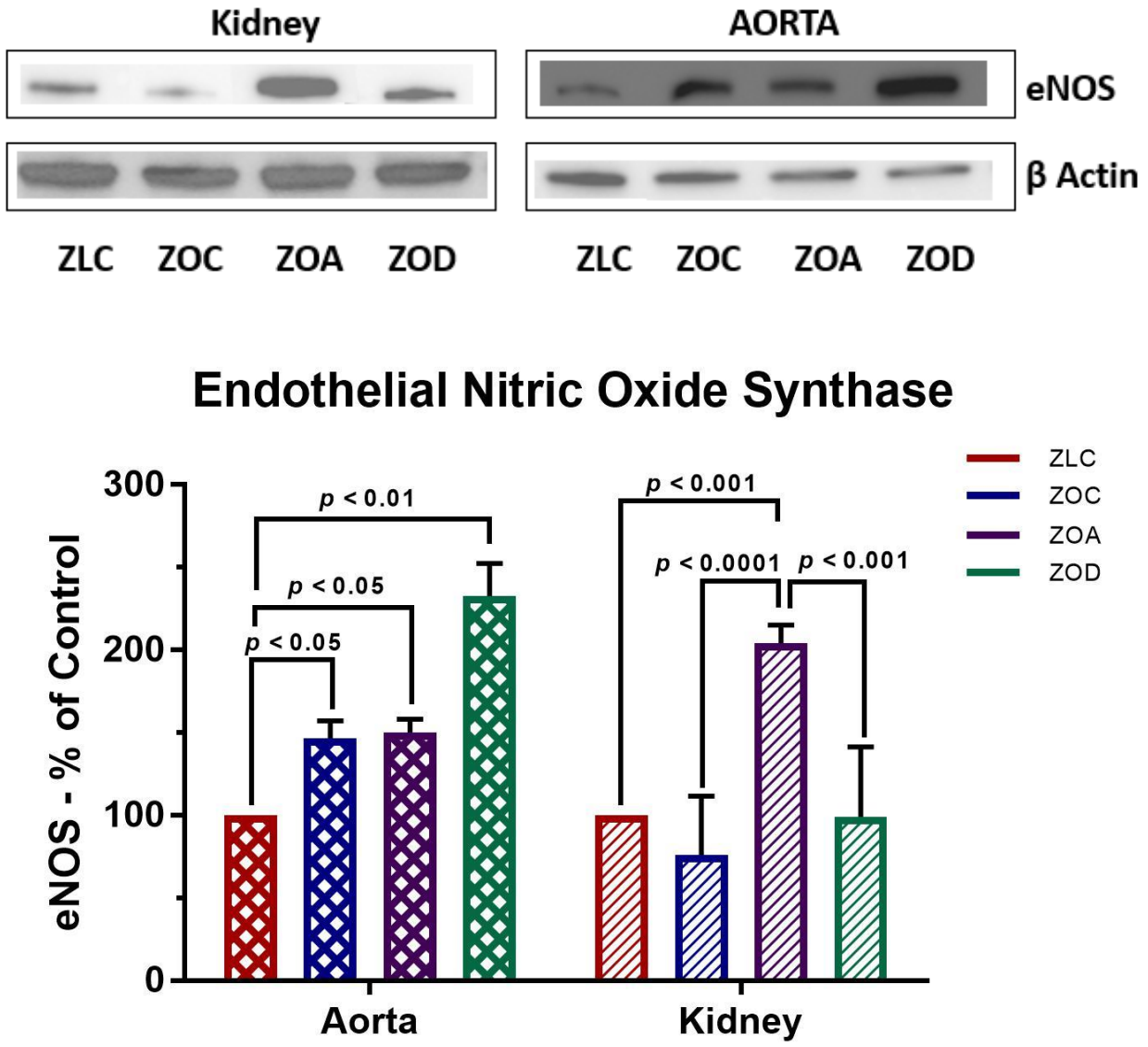

Figure 3.17. Effect of oral arginine (1000 mg/kg/day) for 12 weeks on the expression of endothelial nitric oxide synthase in the aorta, kidney and brain of Zucker lean and Zucker Diabetic Fatty rats.

6-week-old male Zucker lean control (ZLC) and Zucker Diabetic Fatty control (ZOC) rats were given normal drinking water for 12 weeks. An additional group of Zucker Diabetic Fatty rats were treated with L-Arginine (ZOA) or D-Arginine (ZOD) in drinking water (1000 mg/kg/day) for 12 weeks. Western blotting was used to quantify eNOS expression in the aorta and kidney. The blots were quantified using the image analysis program GeneTools from Syngene (Aorta, n = 6-10; Kidney, n = 5-6).

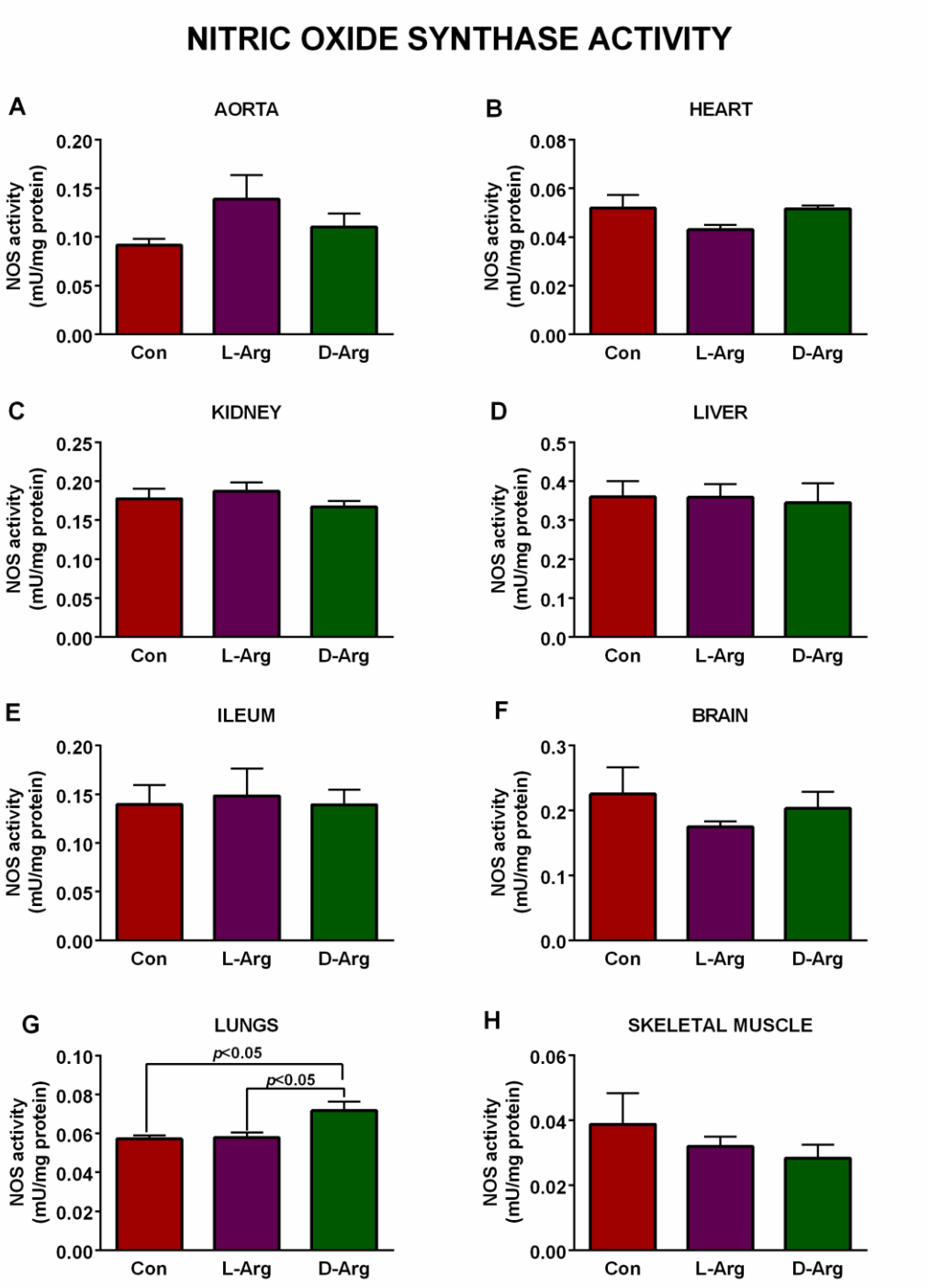

Figure 3.18. Effect of oral arginine (500 mg/kg/day) for 4 weeks on nitric oxide synthase activity in different organs/tissues of Sprague Dawley rats.

10-week-old male Sprague Dawley rats were treated with L-Arginine (L-Arg) or D-Arginine (D-Arg) in drinking water (500 mg/kg/day) for 4 weeks. An assay kit was used to measure NOS activity (Control, n = 4-8; L-Arg, n = 5-9; D-Arg, n = 6-8).

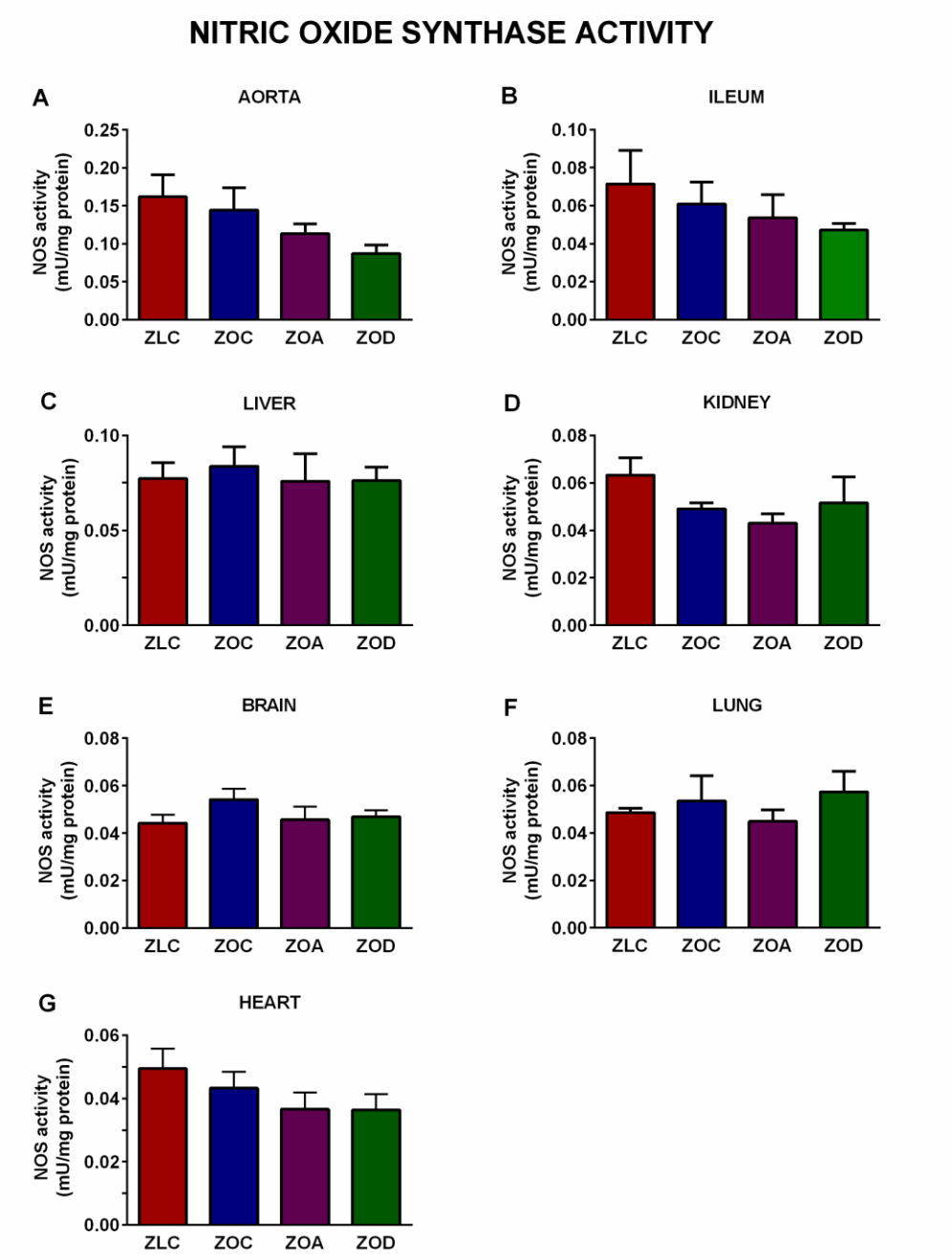

Figure 3.19. Effect of oral arginine (1000 mg/kg/day) for 12 weeks on nitric oxide synthase activity in plasma and different organs/tissues of Zucker rats.

6-week-old male Zucker lean control (ZLC) and Zucker Diabetic Fatty control (ZOC) rats were given normal drinking water for 12 weeks. An additional group of Zucker Diabetic Fatty rats were treated with L-Arginine (ZOA) or D-Arginine (ZOD) in drinking water (1000 mg/kg/day) for 12 weeks. An assay kit was used to measure NOS activity (ZLC, *n* = 5; ZOC, *n* = 4-5; ZOA, *n* = 5; ZOD, *n* = 4-5).

3.23 Oral L-Arginine supplements (500 mg/kg/day) significantly increased plasma NOx levels while oral D-Arginine (500 mg/kg/day) significantly decreased plasma NO_x levels of Sprague Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 4 weeks with 500 mg/kg/day of L-Arg significantly increased NOx levels in the plasma (Fig. 3.20) compared to control. Treatment with D-Arg significantly decreased plasma NOx levels as compared to L-Arg (Fig. 3.20). Neither L nor D-Arg significantly altered NOx levels in the aorta, lungs, kidney, liver, brain, skeletal muscle or ileum (Fig. 3.20).

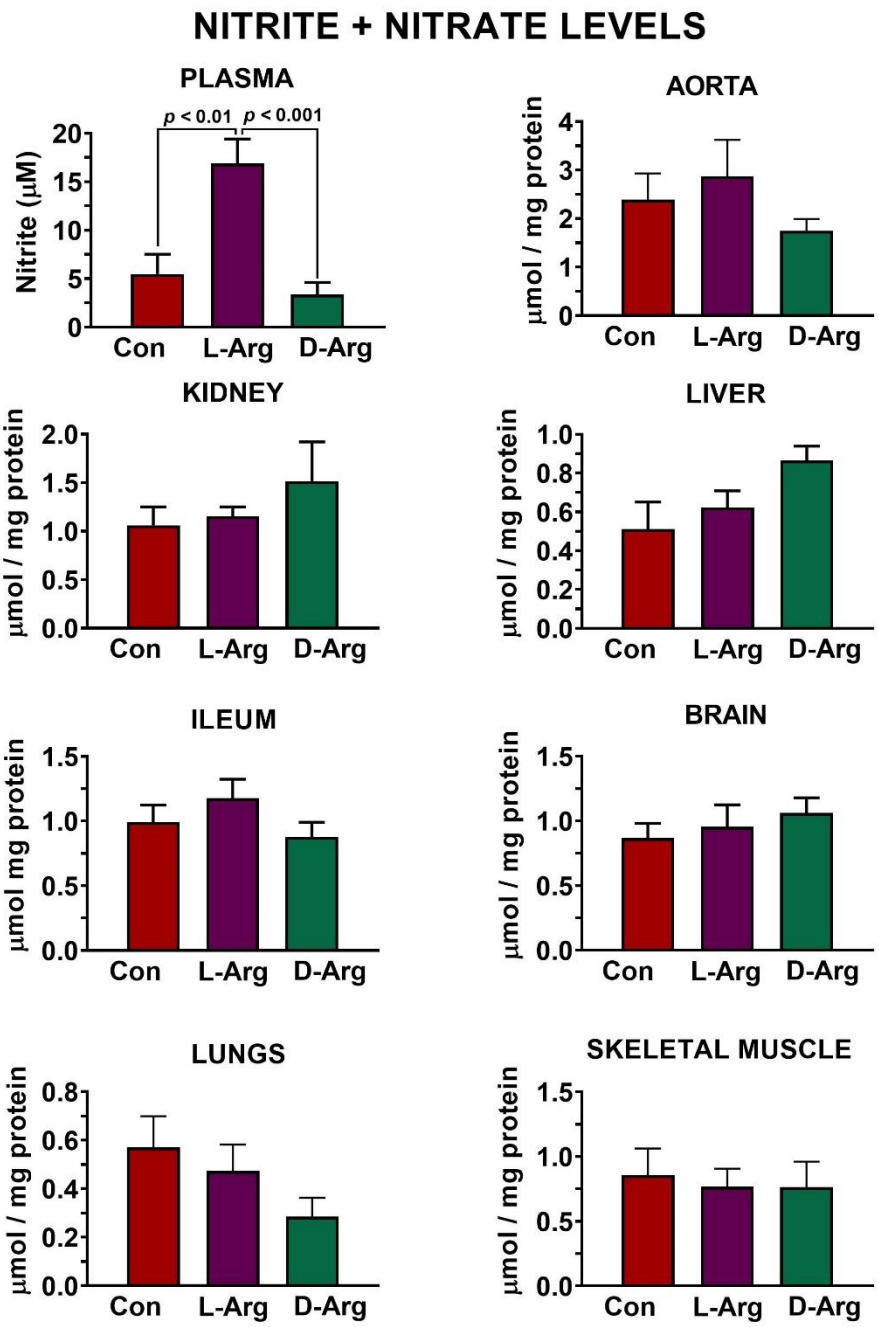

Figure 3.20. Effect of oral arginine (500 mg/kg/day) for 4 weeks on NOx (an indicator of nitric oxide production) levels in plasma and different organs/tissues of Sprague Dawley rats.

10-week-old male Sprague Dawley rats were treated with L-Arginine (L-Arg) or D-Arginine (D-Arg) in drinking water (500 mg/kg/day) for 4 weeks. An assay kit with Griess reagent was used to measure nitrate + nitrite levels (Control, *n* = 6-8; L-Arg, *n* = 7-9*;* D-Arg, *n* = 6-8).

3.24 Oral arginine supplements (1000 mg/kg/day) significantly decreased NOx levels in the liver, skeletal muscle, and lung of Zucker Diabetic Fatty Rats.

Treatment of 6-week-old male Zucker Diabetic Fatty rats with 1000 mg/kg/day of L- or D-Arg resulted in significantly decreased NOx levels in the liver as compared to lean controls (Fig. 3.21). Additionally, Zucker obese controls had significantly decreased kidney NOx levels as compared to lean controls (Fig. 3.21). L-Arg supplementation also significantly decreased NOx levels in the skeletal muscle as compared to Zucker obese controls. In the lung, D-Arg significantly decreased NOx levels as compared to the lean control (Fig. 3.21). Neither isomer significantly affected NOx levels in the plasma, aorta, ileum or brain (Fig. 3.21). Overall, NO_x levels were lower in ZDF rats as they were measured on a scale of nmol/mg of protein (Fig. 3.21) *vs* SD rats in which levels were measure on a scale of µmol/mg of protein (Fig. 3.20). This could possibly be due to the use of different assay kits, *viz*. colorimetric *vs*. flurometric in the two assays.

3.25 Oral arginine supplements (500 mg/kg/day) significantly decreased arginase expression in the ileum and liver of Sprague Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 4 weeks with 500 mg/kg/day of L-Arg or D-Arg significantly decreased arginase expression in the ileum as compared to control (Fig. 3.22). D-Arg also significantly decreased arginase expression in the liver as compared to control (Fig. 3.22). Treatment with L-Arg had no significant effect on arginase expression in the liver and neither isomer affected kidney arginase as compared to control (Fig. 3.22).

3.26 Oral arginine supplements (1000 mg/kg/day) significantly increased arginase expression in the ileum of Zucker Diabetic Fatty rats without affecting arginase expression in the liver or kidney.

6-week-old male Zucker Diabetic Fatty control rats given normal drinking water had significantly increased arginase expression in the ileum with no change in the liver or kidney as compared to Zucker lean controls (Fig. 3.23). Treatment of 6-week-old male Zucker Diabetic Fatty rats with 1000 mg/kg/day of L- or D-Arg also significantly increased arginase expression in the ileum as compared to Zucker lean controls (Fig. 3.23). Neither isomer affected arginase expression in the liver or kidney (Fig. 3.23).

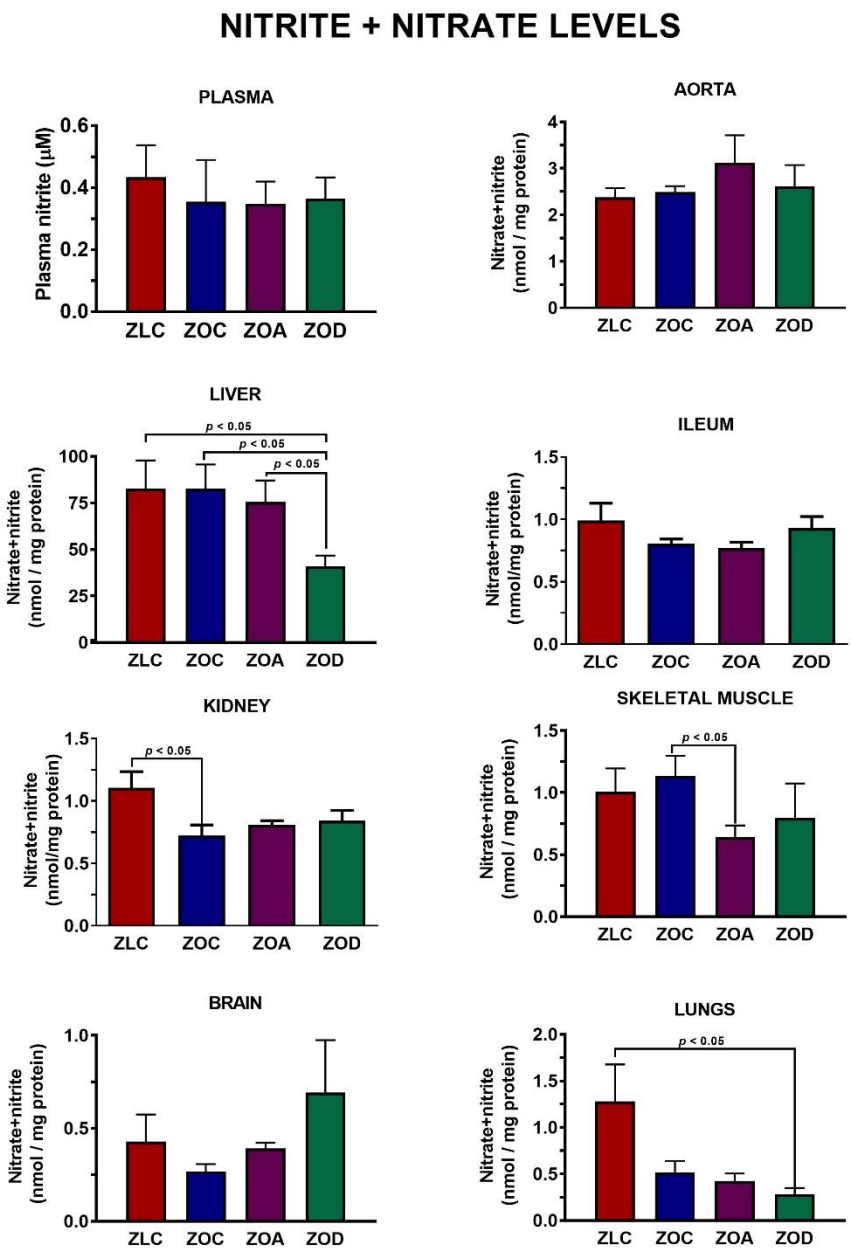

Figure 3.21. Effect of oral arginine (1000 mg/kg/day) for 12 weeks on NOx (an indicator of nitric oxide production) levels in plasma and different organs/tissues of Zucker rats.

6-week-old male Zucker lean control (ZLC) and Zucker Diabetic Fatty control (ZOC) rats were given normal drinking water for 12 weeks. An additional group of Zucker Diabetic Fatty rats were treated with L-Arginine (ZOA) or D-Arginine (ZOD) in drinking water (1000 mg/kg/day) for 12 weeks. An assay kit with Griess reagent was used to measure nitrate + nitrite levels (ZLC, n = 5; ZOC, n = 4-5; ZOA, n = 5; ZOD, n = 4-5).

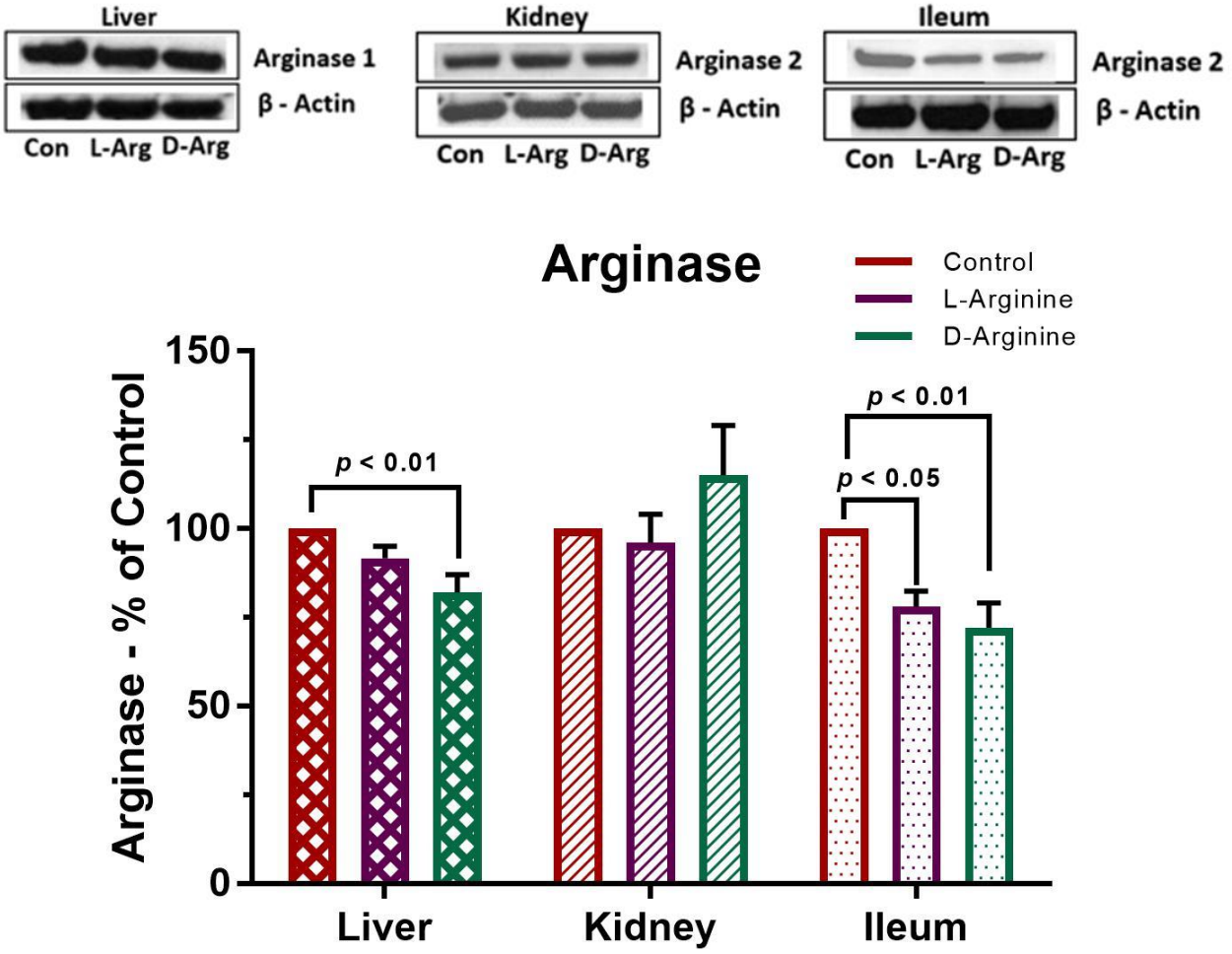

Figure 3.22. Effect of oral arginine (500 mg/kg/day) for 4 weeks on the expression of arginases in the liver, kidney and ileum of Sprague Dawley rats.

10-week-old male Sprague Dawley rats were treated with L-Arginine (L-Arg) or D-Arginine (D-Arg) in drinking water (500 mg/kg/day) for 4 weeks. Western blotting was used to quantify arginase I and II expression in the liver, kidney and ileum. The blots were quantified using the image analysis program GeneTools from Syngene (Liver, n = 13; Kidney, n = 17; Ileum, n = 6).

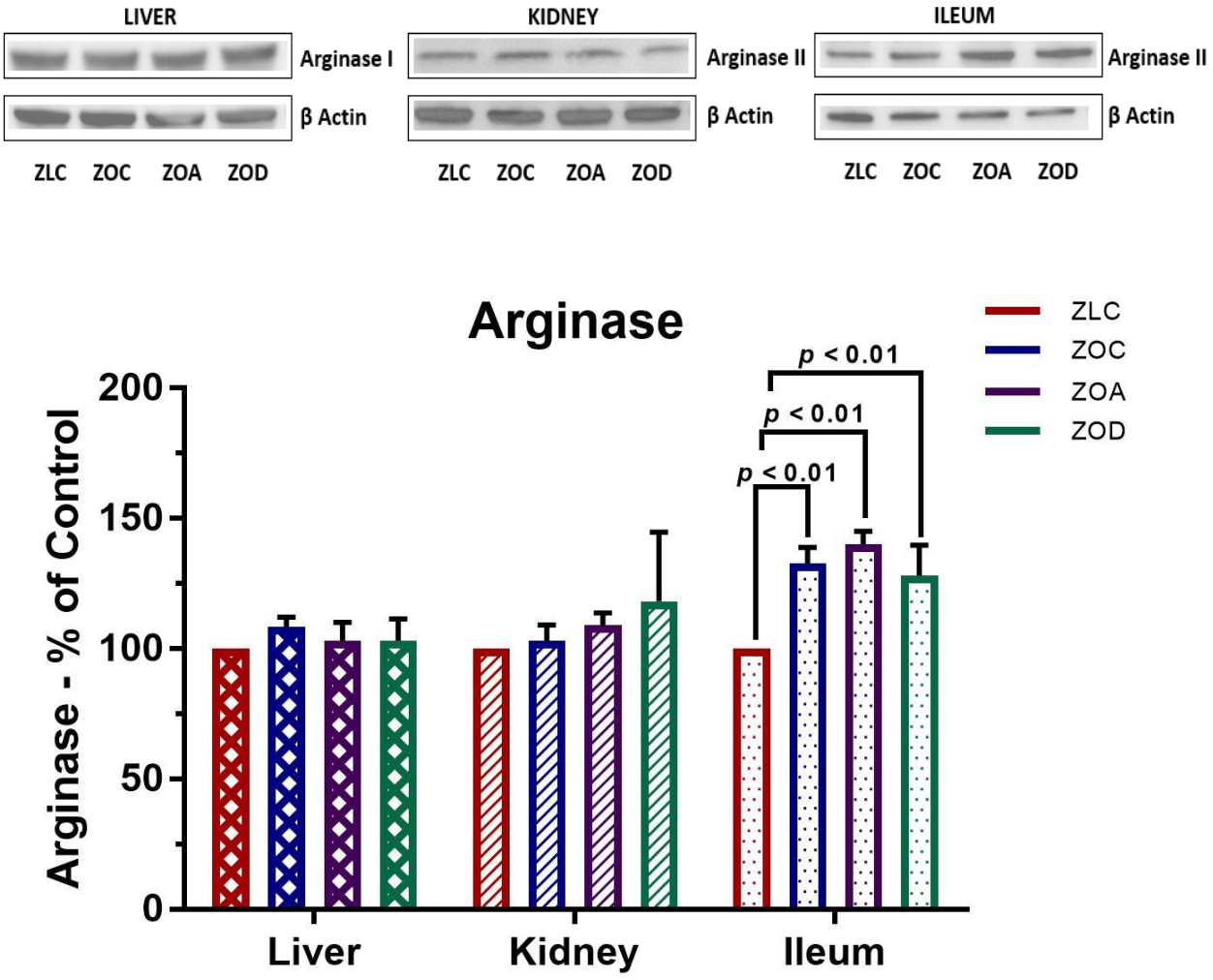

Figure 3.23. Effect of oral arginine (1000 mg/kg/day) for 12 weeks on the expression of arginases in the liver, kidney and ileum of Zucker rats.

6-week-old male Zucker lean control (ZLC) and Zucker Diabetic Fatty control (ZOC) rats were given normal drinking water for 12 weeks. An additional group of Zucker Diabetic Fatty rats were treated with L-Arginine (ZOA) or D-Arginine (ZOD) in drinking water (1000 mg/kg/day) for 12 weeks. Western blotting was used to quantify arginase I and II expression in the liver, kidney and ileum. The blots were quantified using the image analysis program GeneTools from Syngene (Liver, n = 3; Kidney, n = 3-6; Ileum, n = 3-4).

3.27 Oral L- and D-Arginine supplements (500 mg/kg/day) did not significantly alter arginase activity in the plasma, liver, kidney or ileum of Sprague Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 4 weeks with 500 mg/kg/day of L or D-Arg did no significantly alter activity of arginase in the plasma, liver, kidney, or ileum as compared to control (Fig. 3.24).

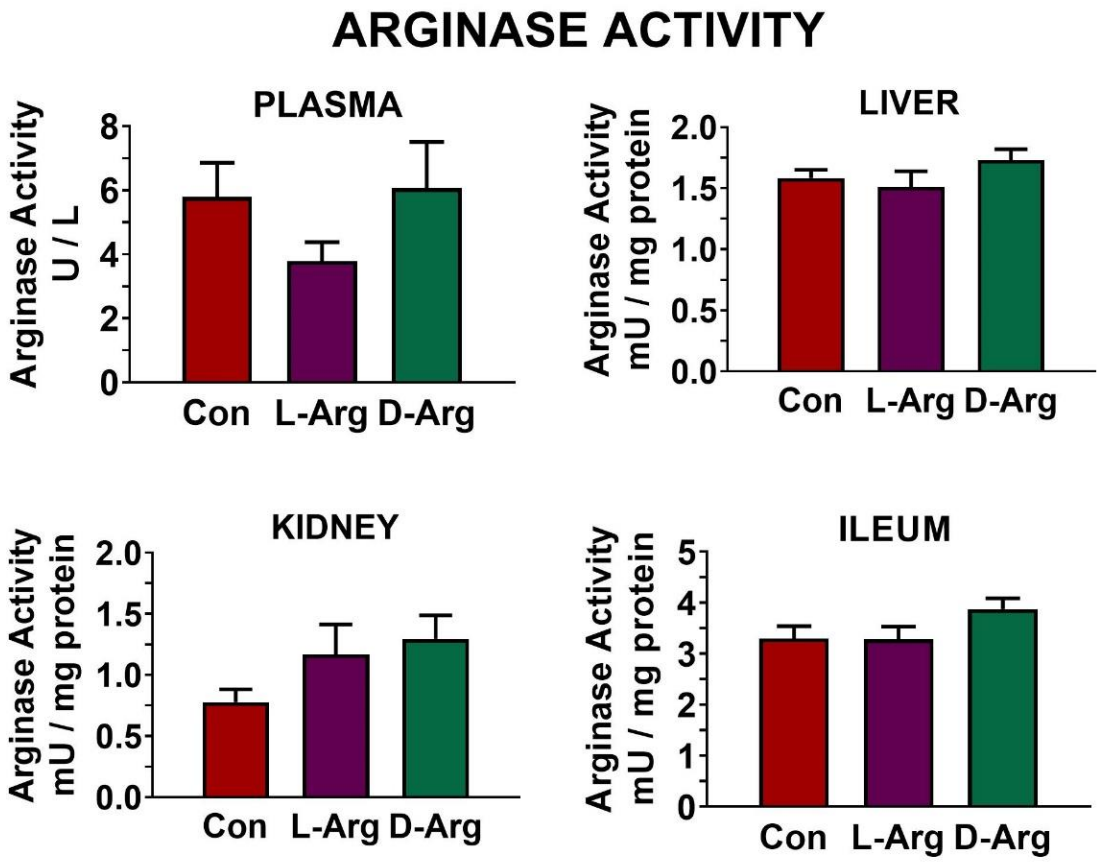

Figure 3.24. Effect of oral arginine (500 mg/kg/day) for 4 weeks on arginase activity in the plasma and different organs/tissues of Sprague Dawley rats.

Ten-week-old male Sprague Dawley rats were treated with L-Arginine (L-Arg) or D-Arginine (D-Arg) in drinking water (500 mg/kg/day) for 4 weeks. An assay kit was used to measure arginase activity (Control, n = 7-8; L-Arg, n = 8-9; D-Arg, n = 7-8).

3.28 Oral L-arginine (1000 mg/kg/day) for 12 weeks significantly decreased arginase activity in the plasma and ileum of Zucker Diabetic Fatty rats.

Treatment of 6-week-old Zucker Diabetic Fatty rats for 12 weeks with 1000 mg/kg/day of L-Arg, but not D-Arg, significantly decreased plasma arginase activity as compared to obese controls (Fig. 3.25). In the ileum, both L- and D-Arg significantly decreased arginase activity as compared to lean controls (Fig. 3.25). This decrease was also observed in the ileum of obese controls as compared to lean controls (Fig. 3.25).

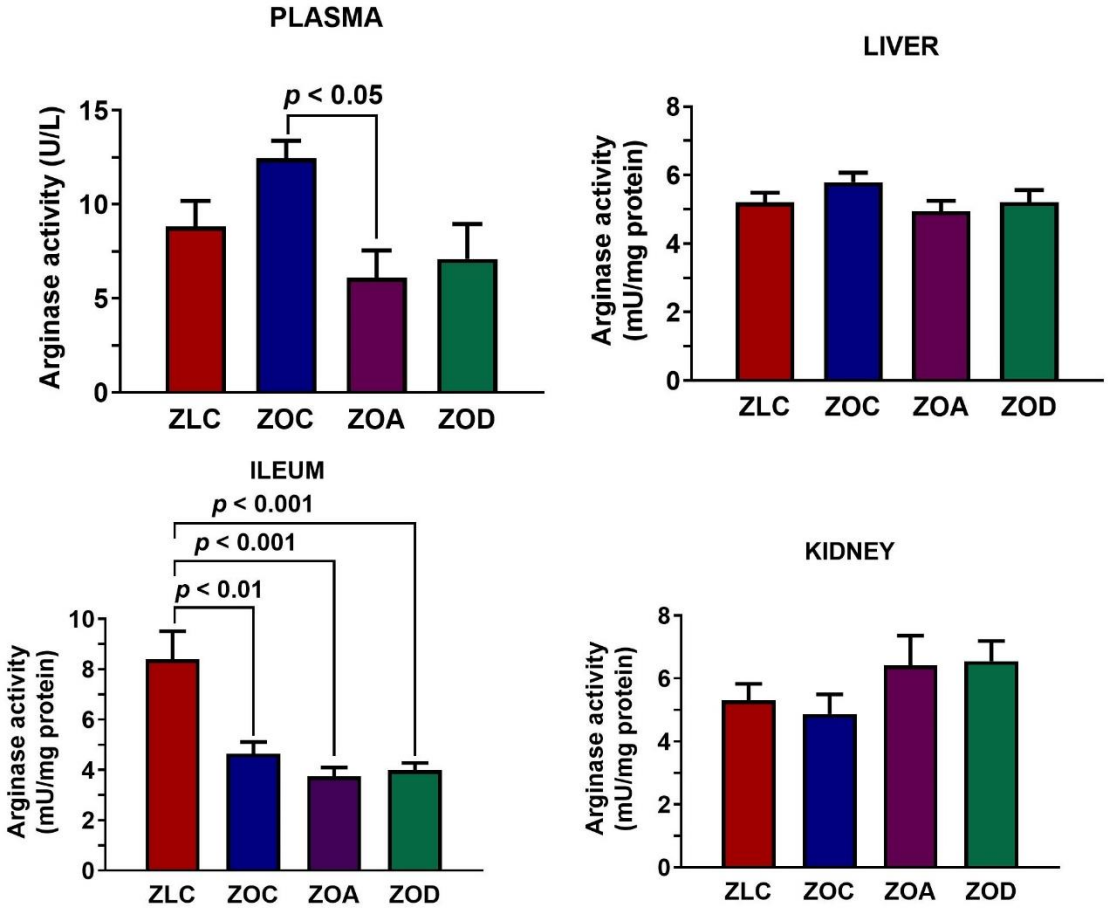

Figure 3.25. Effect of oral arginine (1000 mg/kg/day) for 12 weeks on arginase activity in the plasma and different organs/tissues of Zucker lean and Zucker Diabetic Fatty rats.

6-week-old male Zucker lean control (ZLC) and Zucker Diabetic Fatty control (ZOC) rats were given normal drinking water for 12 weeks. An additional group of Zucker Diabetic Fatty rats were treated with L-Arginine (ZOA) or D-Arginine (ZOD) in drinking water (1000 mg/kg/day) for 12 weeks. An assay kit was used to measure arginase activity (ZLC, n = 5; ZOC, n = 4-5; ZOA, n = 5; ZOD, n = 4-5).

3.29 Oral D-Arginine supplements (500 mg/kg/day) but not oral L-Arginine supplements significantly increased urea levels in the liver and kidney of Sprague Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 4 weeks with 500 mg/kg/day of D-Arg significantly increased liver and kidney urea levels as compared to control (Fig. 3.26). D-Arg also significantly increased urea levels in the kidney as compared to the L-Arg group (Fig. 3.26). Treatment with L-Arg did not significantly alter urea levels in the liver or kidney as compared to control (Fig. 3.26).

3.30 Oral L- and D-Arginine supplements (500 mg/kg/day) do not significantly alter urea levels in the plasma, urine, lungs, brain or skeletal muscle of Sprague Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 4 weeks with 500 mg/kg/day of L- or D-Arg did not significantly alter urea levels in the plasma, urine, lungs, brain or skeletal muscle as compared to control (Fig. 3.26). Overall, urea levels are higher in SD rats (Fig. 3.26) *vs* ZDF rats (Fig. 3.27) and thus a larger scale of µmol/mg of protein was used.

3.31 Oral arginine (1000 mg/kg/day) for 12 weeks on urea significantly increased plasma urea levels but had no effect in the skeletal muscle, liver brain or urine of Zucker Diabetic Fatty rats.

Treatment of 6-week-old male Zucker Diabetic Fatty rats with 1000 mg/kg/day for 12 weeks of L- or D-Arg significantly increased plasma urea levels as compared to Zucker lean controls (Fig. 3.27). Zucker obese controls also had significantly increased plasma urea levels as compared to Zucker lean controls (Fig. 3.27 Neither L- or D-Arg supplements significantly altered urea levels in the skeletal muscle, liver, brain or urine (Fig. 3.27).

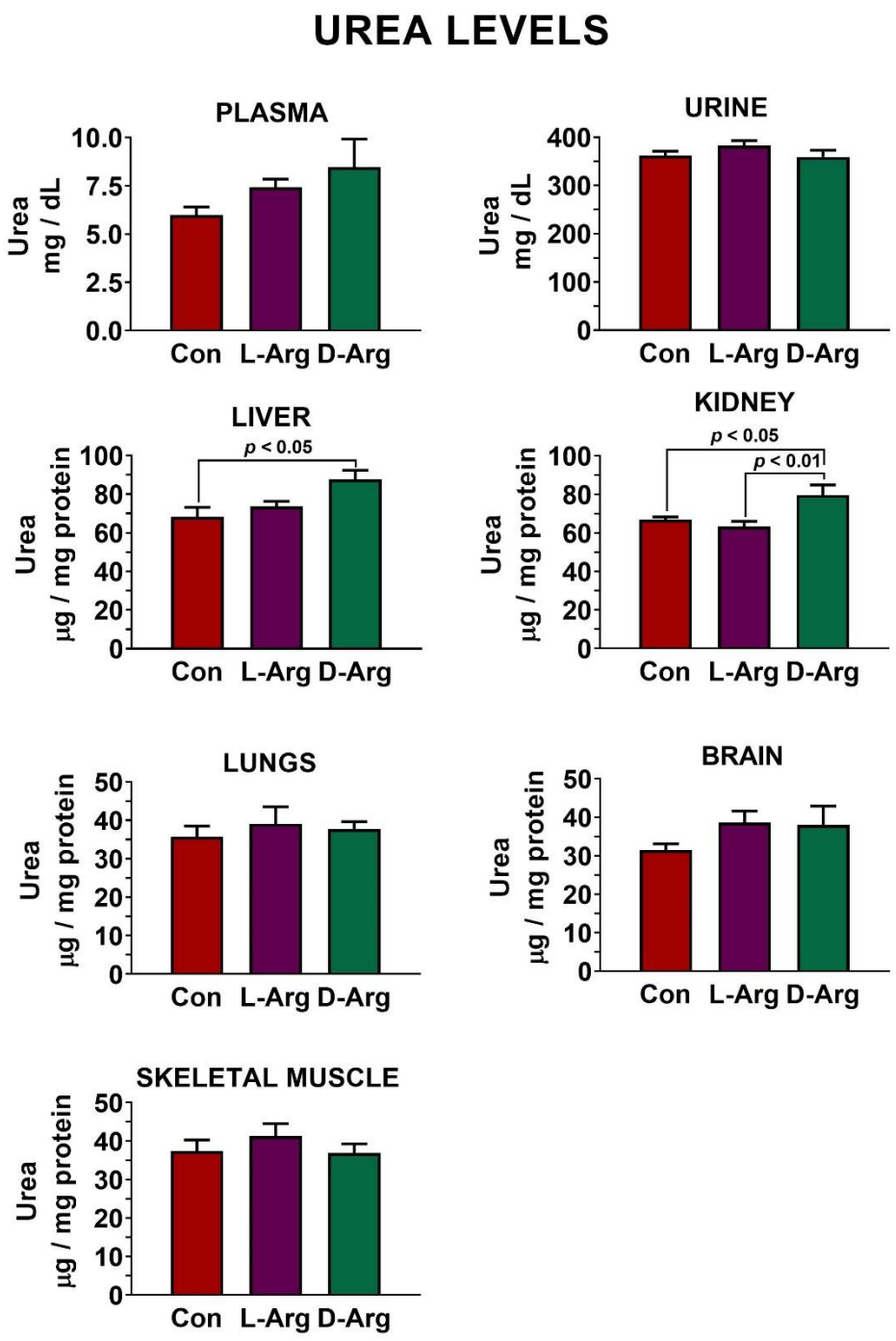

Figure 3.26. Effect of oral arginine (500 mg/kg/day) for 4 weeks on urea (a metabolic product of arginases) levels in plasma and different organs/tissues of Sprague Dawley rats.

10-week-old male Sprague Dawley rats were treated with L-Arginine (L-Arg) or D-Arginine (D-Arg) in drinking water (500 mg/kg/day) for 4 weeks. An assay kit was used to measure urea levels (Control, n = 7-8; L-Arg, n = 9; D-Arg, n = 8).

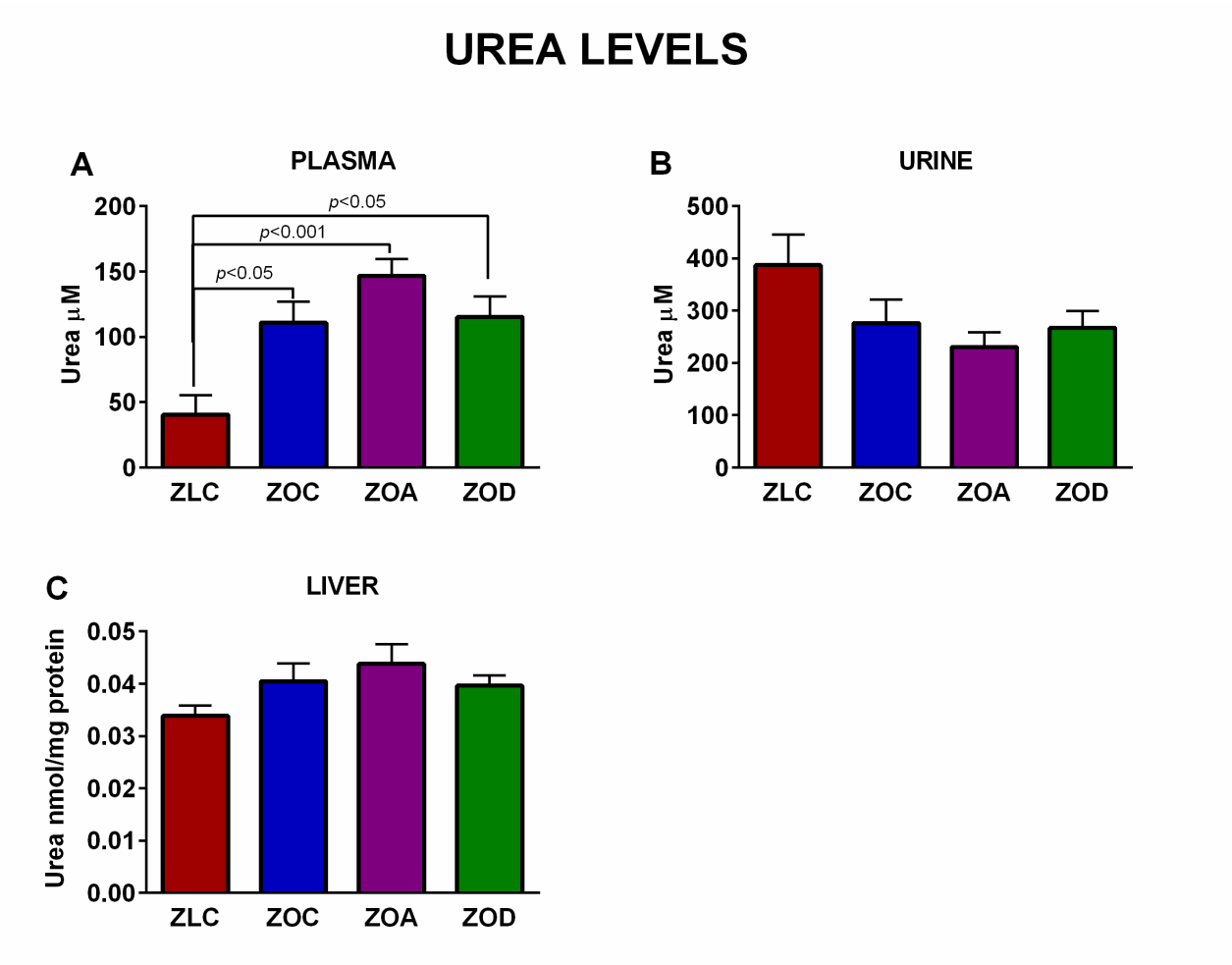

Figure 3.27. Effect of oral arginine (1000 mg/kg/day) for 12 weeks on urea (a metabolic product of arginases) in plasma and different organs/tissues of Zucker rats.

6-week-old male Zucker lean control (ZLC) and Zucker Diabetic Fatty control (ZOC) rats were given normal drinking water for 12 weeks. An additional group of Zucker Diabetic Fatty rats were treated with L-Arginine (ZOA) or D-Arginine (ZOD) in drinking water (1000 mg/kg/day) for 12 weeks. An assay kit was used to measure urea levels (ZLC, *n* = 5; ZOC, *n* = 4-5; ZOA, *n* = 5; ZOD, *n* = 4-5). (Note: A different type of assay kit was used for this assay than in Fig. 3.26)

3.32 Oral L-Arginine supplements (500 mg/kg/day) significantly increased arginine decarboxylase expression in the liver but not in the brain of Sprague Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 4 weeks with 500 mg/kg/day of L-Arg significantly increased arginine decarboxylase expression in the liver as compared to control (Fig. 3.28). L-Arg did not have any significant effect on arginine decarboxylase expression in the brain as compared to control. Treatment with D-Arg had no significant effect on arginine decarboxylase expression in the liver or brain as compared to control (Fig. 3.28).

3.33 Oral D-Arginine supplements (500 mg/kg/day) significantly increased arginine decarboxylase levels in the ileum but had no effect on the plasma, kidney, liver or brain of Sprague Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 4 weeks with 500 mg/kg/day of D-Arg significantly increased arginine decarboxylase protein levels in the ileum as compared control (Fig. 3.29). D-Arginine treatment did not have any significant effect on arginine decarboxylase protein levels in the plasma, kidney, liver and brain compared to control (Fig. 3.29). Treatment with L-Arg also did not significantly alter arginine decarboxylase levels in the plasma or in these 4 organs (Fig. 3.29).

3.34 Oral D-Arginine supplements (500 mg/kg/day) significantly decreased agmatinase expression in the liver but not the kidney, ileum or brain of Sprague Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 4 weeks with 500 mg/kg/day of D-Arg significantly decreased agmatinase expression in the liver as compared to control (Fig. 3.30). L-Arg did not have any significant effect on agmatinase expression in the liver, kidney, ileum or brain as compared to control (Fig.3.30). Treatment with D-Arg had no significant effect on agmatinase expression in the kidney, ileum or brain as compared to control (Fig. 3.30).

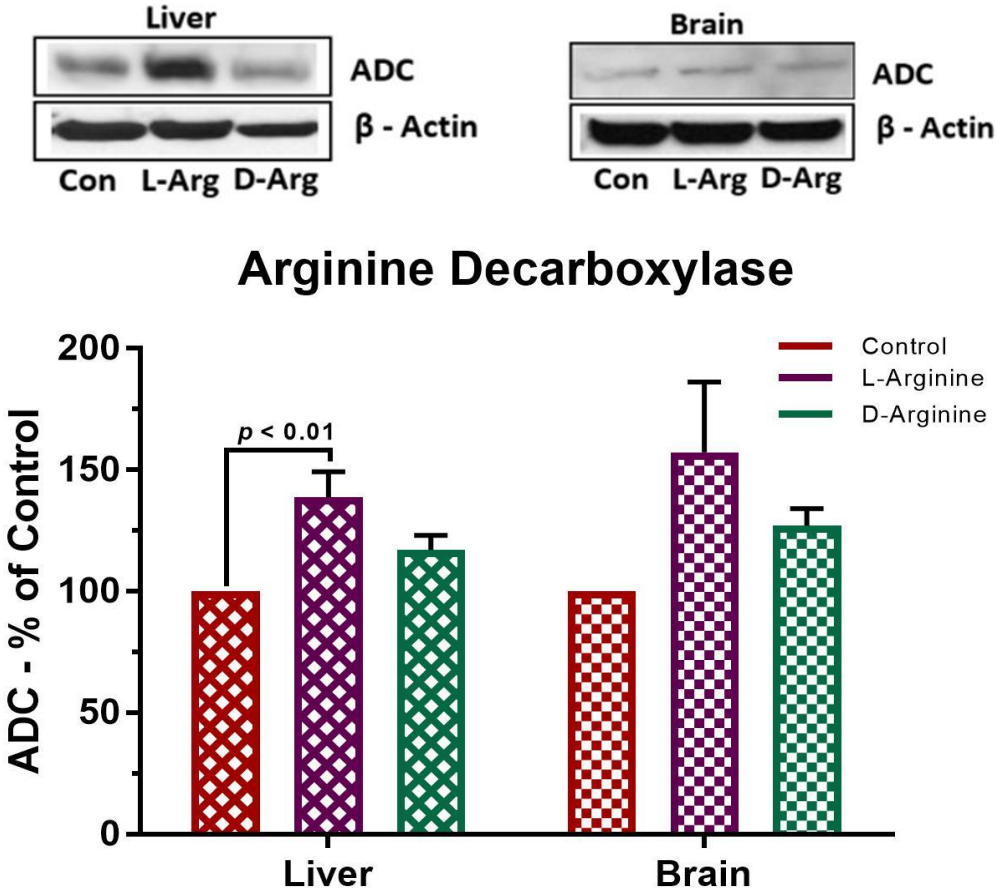

Figure 3.28. Effect of oral arginine (500 mg/kg/day) for 4 weeks on the expression of arginine decarboxylase in the liver and brain of Sprague Dawley rats.

10-week-old male Sprague Dawley rats were treated with L-Arginine (L-Arg) or D-Arginine (D-Arg) in drinking water (500 mg/kg/day) for 4 weeks. Western blotting was used to quantify arginase I and II expression in the liver, kidney and ileum. The blots were quantified using the image analysis program GeneTools from Syngene (Liver, n = 6, Brain, n = 3).

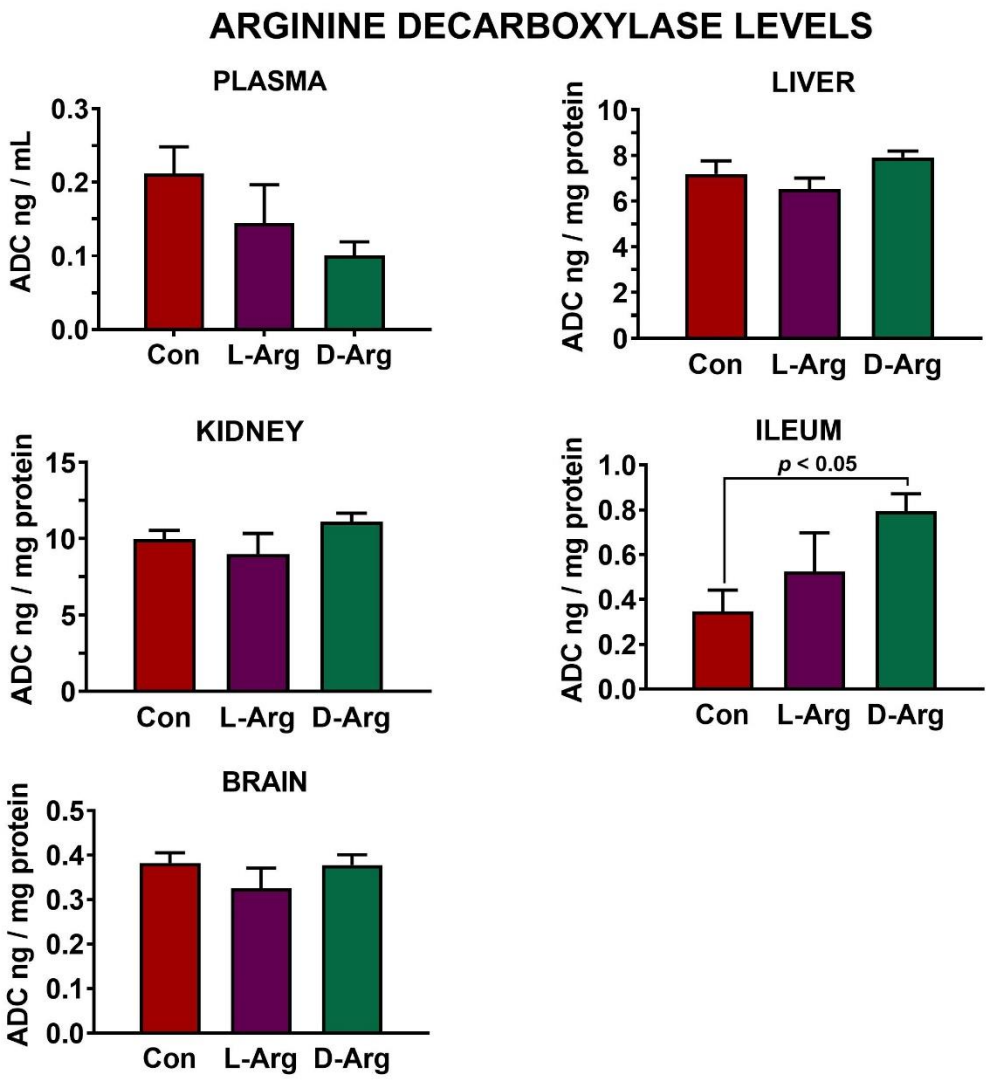

Figure 3.29. Effect of oral arginine (500 mg/kg/day) for 4 weeks on arginine decarboxylase levels in the plasma, liver, brain, kidney and ileum of Sprague Dawley rats.

10-week-old male Sprague Dawley rats were treated with L-Arginine (L-Arg) or D-Arginine (D-Arg) in drinking water (500 mg/kg/day) for 4 weeks. An assay kit was used to quantify the concentration of ADC present (Control, n = 4-5; L-Arg, n = 4-5; D-Arg, n = 4-5).

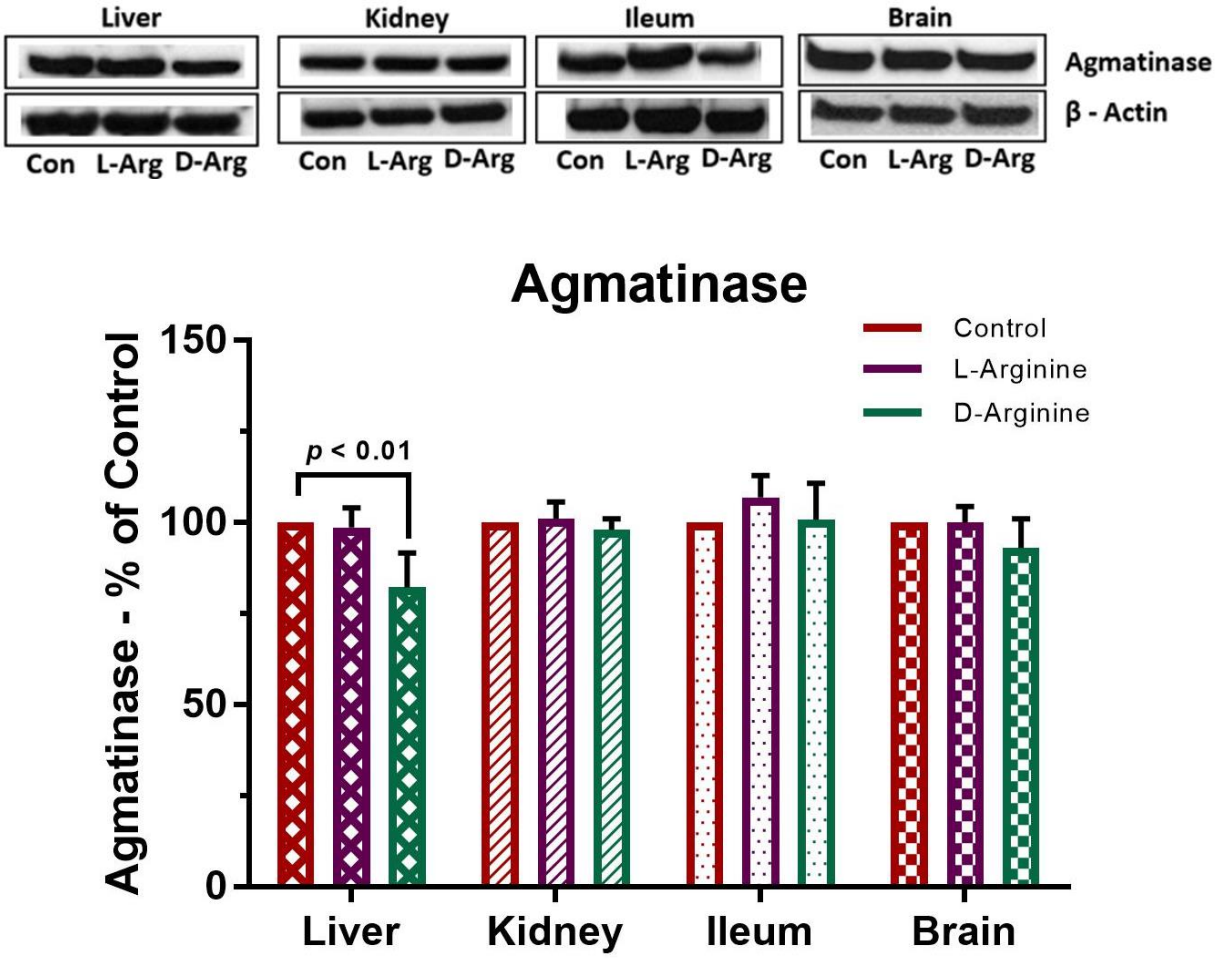

Figure 3.30. Effect of oral arginine (500 mg/kg/day) for 4 weeks on the expression of agmatinase in the liver, ileum, kidney and brain of Sprague Dawley rats.

10-week-old male Sprague Dawley rats were treated with L-Arginine (L-Arg) or D-Arginine (D-Arg) in drinking water (500 mg/kg/day) for 4 weeks. Western blotting was used to quantify ADC expression in the liver, kidney, ileum and brain. The blots were quantified using the image analysis program GeneTools from Syngene (Liver, n = 5; Kidney, n = 9; Ileum, n = 8-10; Brain, n = 5-6).

3.35 Oral D-Arginine supplements (500 mg/kg/day) significantly increased polyamines in the plasma but significantly decreased polyamines in the liver of Sprague Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 4 weeks with 500 mg/kg/day of D-Arg significantly increased polyamines (metabolites of arginine decarboxylase and agmatinase) in the plasma as compared to control but significantly decreased polyamines in the liver as compared to L-Arg (Fig. 3.31). D-Arg did not have any significant effect on polyamine levels in the kidney, ileum and brain (Fig. 3.31).

3.36 Oral L-Arginine supplements (500 mg/kg/day) significantly increased polyamines in the liver but had no effect on polyamines in the plasma, kidney, ileum and brain of Sprague Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 4 weeks with 500 mg/kg/day of L-Arg significantly increased polyamines in the liver as compared to control (Fig. 3.31). L-Arg did not have any significant effect on polyamine levels in the plasma, kidney, ileum and brain (Fig. 3.31).

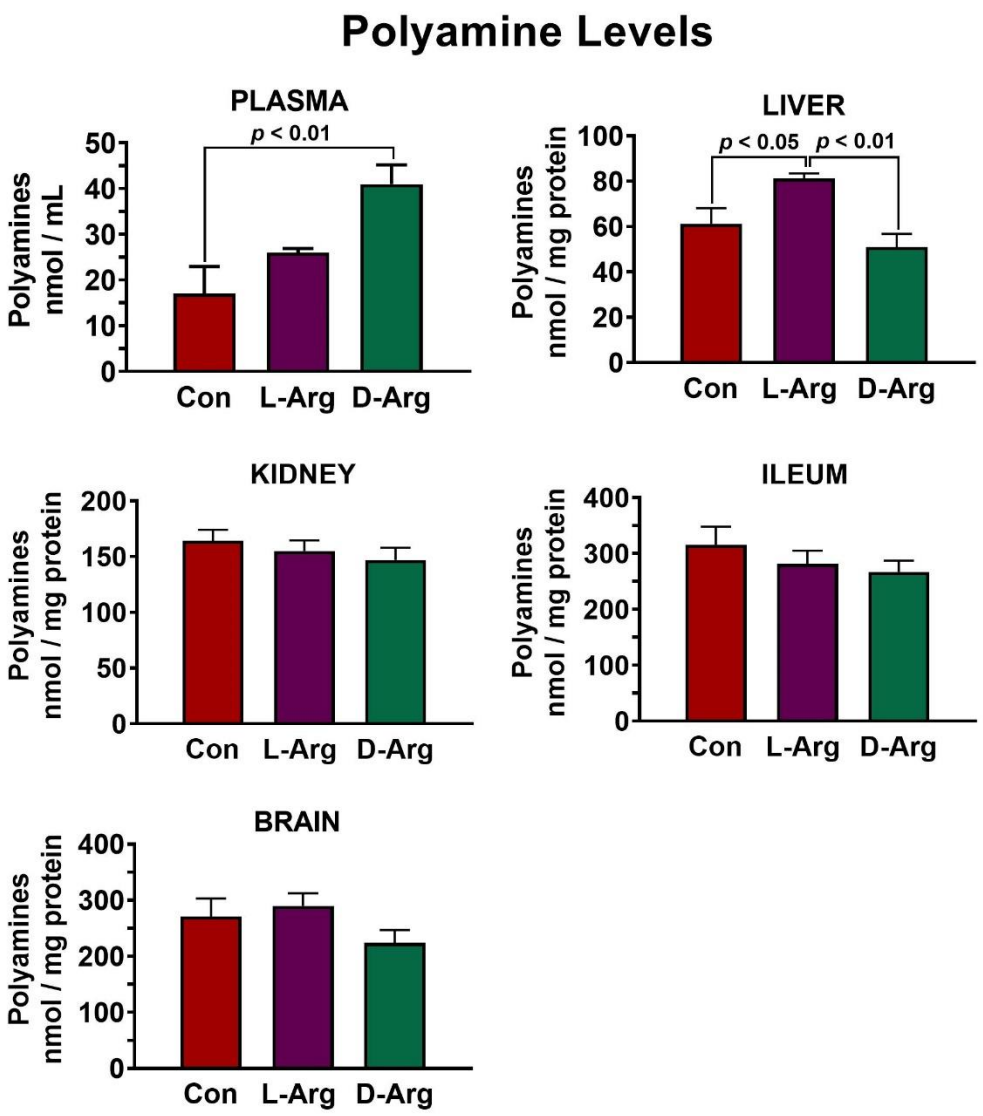

Figure 3.31. Effect of oral arginine (500 mg/kg/day) for 4 weeks on polyamines levels in the plasma and different organs/tissues of Sprague Dawley rats.

10-week-old male Sprague Dawley rats were treated with L-Arginine (L-Arg) or D-Arginine (D-Arg) in drinking water (500 mg/kg/day) for 4 weeks. An assay kit was used to quantify polyamine levels (Control, *n* = 3-6; L-Arg, *n* = 3-8*;* D-Arg, *n* = 5-6).

3.37 Oral L-Arginine supplements (500 mg/kg/day) significantly decreased guanidinoacetate methyltransferase (AGAT) expression in the liver and kidney but has no effect on AGAT expression in the ileum or brain of Sprague Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 4 weeks with 500 mg/kg/day of L-Arg significantly decreased arginine: glycine amidinotransferase expression in the liver and kidney as compared to control (Fig. 3.32). Treatment with L-Arg also significantly decreased liver AGAT expression as compared to D-Arg but had no effect on AGAT expression in the ileum or brain as compared to control (Fig. 3.32).

3.38 Oral D-Arginine supplements (500 mg/kg/day) significantly increased arginine: glycine amidinotransferase (AGAT) expression in the kidney but has no effect on AGAT expression in the liver, ileum or brain of Sprague Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 4 weeks with 500 mg/kg/day of D-Arg significantly increased arginine: glycine amidinotransferase expression in the kidney as compared to control and L-Arg (Fig. 3.32). Treatment with D-Arg did not significantly alter AGAT expression in the liver, ileum or brain as compared to control (Fig. 3.32).

3.39 Oral L-Arginine supplements (500 mg/kg/day) significantly decreased creatinine levels in the skeletal muscle with no effect on levels of creatinine in the plasma, urine, liver, kidney or ileum of Sprague Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 4 weeks with 500 mg/kg/day of L-Arg significantly decreased levels of creatinine in the skeletal muscle as compared to control (Fig. 3.33). Treatment with L-Arg did not affect creatinine levels in the plasma, urine, liver, kidney or ileum as compared to control (Fig. 3.33).

3.40 Oral D-Arginine supplements (500 mg/kg/day) significantly increased creatinine levels in the liver with no effect on levels of creatinine in the plasma, urine, skeletal muscle, kidney or ileum of Sprague Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 4 weeks with 500 mg/kg/day of D-Arg significantly increased levels of creatinine in the liver as compared to control (Fig. 3.33). Treatment with D-Arg did not affect creatinine levels in the plasma, urine, skeletal muscle, kidney or ileum as compared to control (Fig. 3.33).

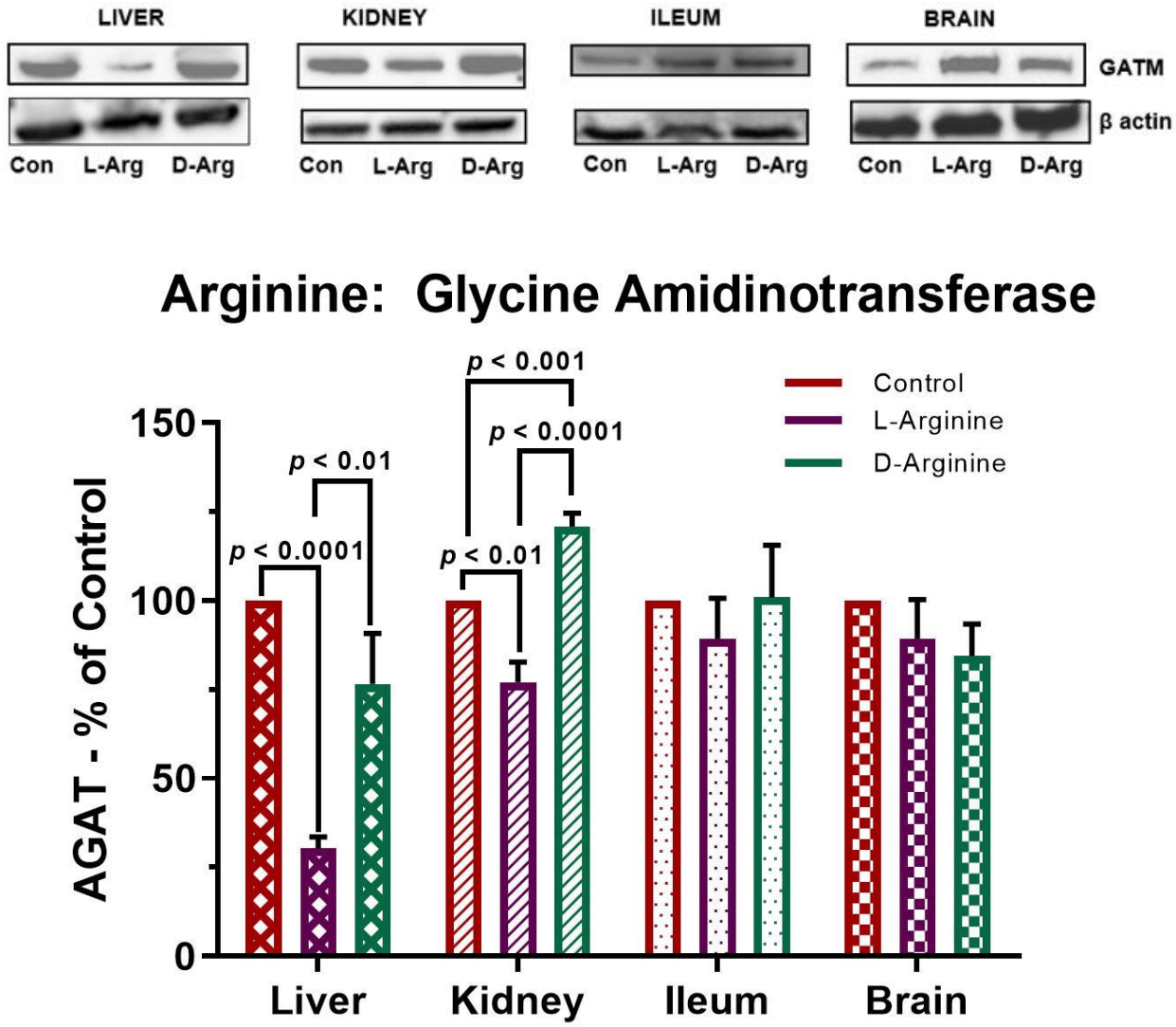

Figure 3.32. Effect of oral arginine (500 mg/kg/day) for 4 weeks on the expression of arginine:glycine amidinotransferase (AGAT) in the liver, kidney, ileum and brain of Sprague Dawley rats.

10-week-old male Sprague Dawley rats were treated with L-Arginine (L-Arg) or D-Arginine (D-Arg) in drinking water (500 mg/kg/day) for 4 weeks. Western blotting was used to quantify AGAT expression in the liver, ileum, kidney and brain. The blots were quantified using the image analysis program GeneTools from Syngene (Liver, n = 6; Kidney, n = 12; Ileum, n = 12; Brain, n = 9).

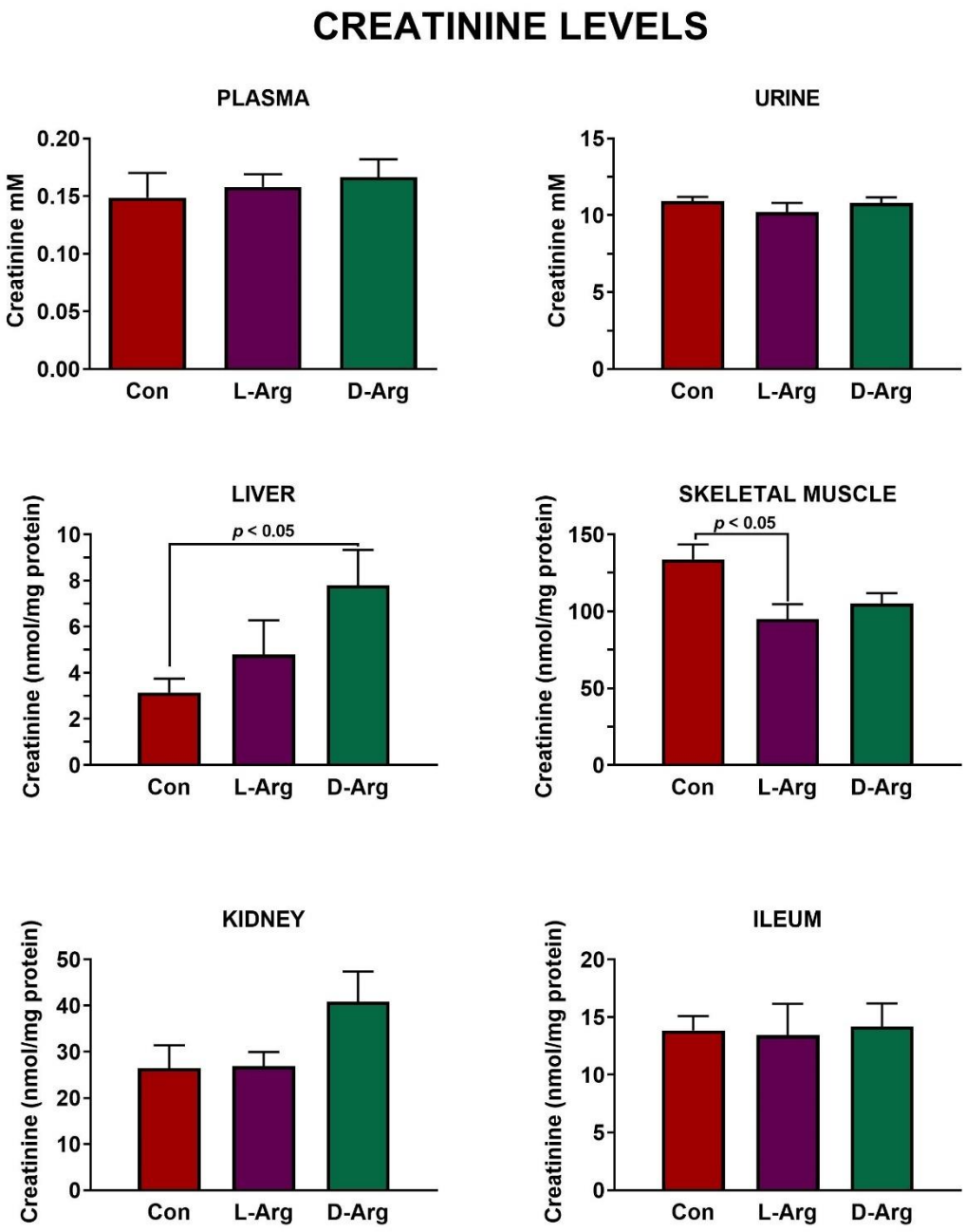

Figure 3.33. Effect of oral arginine (500 mg/kg/day) for 4 weeks on creatinine (a metabolic product of AGAT) levels in the plasma and different organs/tissues of Sprague Dawley rats.

10-week-old male Sprague Dawley rats were treated with L-Arginine (L-Arg) or D-Arginine (D-Arg) in drinking water (500 mg/kg/day) for 4 weeks. An assay kit was used to quantify creatinine levels (Control, n = 7-8; L-Arg, n = 9; D-Arg, n = 7-8).

3.41 Oral L-Arginine supplements (500 mg/kg/day) significantly decreased glyoxalase I expression in the liver, ileum and brain but had no effect on glyoxalase expression in the kidney of Sprague Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 4 weeks with 500 mg/kg/day of L-Arg significantly decreased glyoxalase expression in the liver, ileum and brain as compared to control (Fig. 3.34). Treatment with L-Arg no effect on glyoxalase expression in the kidney as compared to control (Fig. 3.34).

3.42 Oral D-Arginine supplements significantly decreased glyoxalase I expression in the liver and ileum but had no effect on glyoxalase expression in the kidney or brain.

Treatment of 10-week-old male Sprague Dawley rats for 4 weeks with 500 mg/kg/day of D-Arg significantly decreased glyoxalase expression in the liver and ileum as compared to control (Fig. 3.34). Treatment with L-Arg no effect on glyoxalase expression in the kidney or brain as compared to control (Fig. 3.34).

3.43 Oral arginine supplements (1000 mg/kg/day) had no effect on glyoxalase I expression in the liver, ileum or kidney of Zucker rats.

6-week-old Zucker Diabetic Fatty rats given normal drinking water for 12 weeks did not have significantly altered glyoxalase I expression in the liver, ileum or kidney as compared to Zucker lean controls (Fig. 3.35). 6-week-old Zucker Diabetic Fatty rats treated with 1000 mg/kg/day of oral L-Arg or D-Arg for 12 weeks also did not have significantly altered expression of glyoxalase I in the liver, ileum or kidney as compared to Zucker lean controls (Fig. 3.35).

3.44 Oral L and D-Arginine supplements (500 mg/kg/day) significantly increased glyoxalase activity in the brain but had no effect on glyoxalase activity in the liver, kidney or ileum of Sprague Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 4 weeks with 500 mg/kg/day of L-Arg significantly increased glyoxalase activity in the brain as compared to control (Fig. 3.36). Treatment with 500 mg/kg/day of D-Arg also significantly increased glyoxalase activity in the brain as compared to control. Treatment with either L-Arg or D-Arg had no effect on glyoxalase activity in the liver, kidney or ileum (Fig. 3.36).

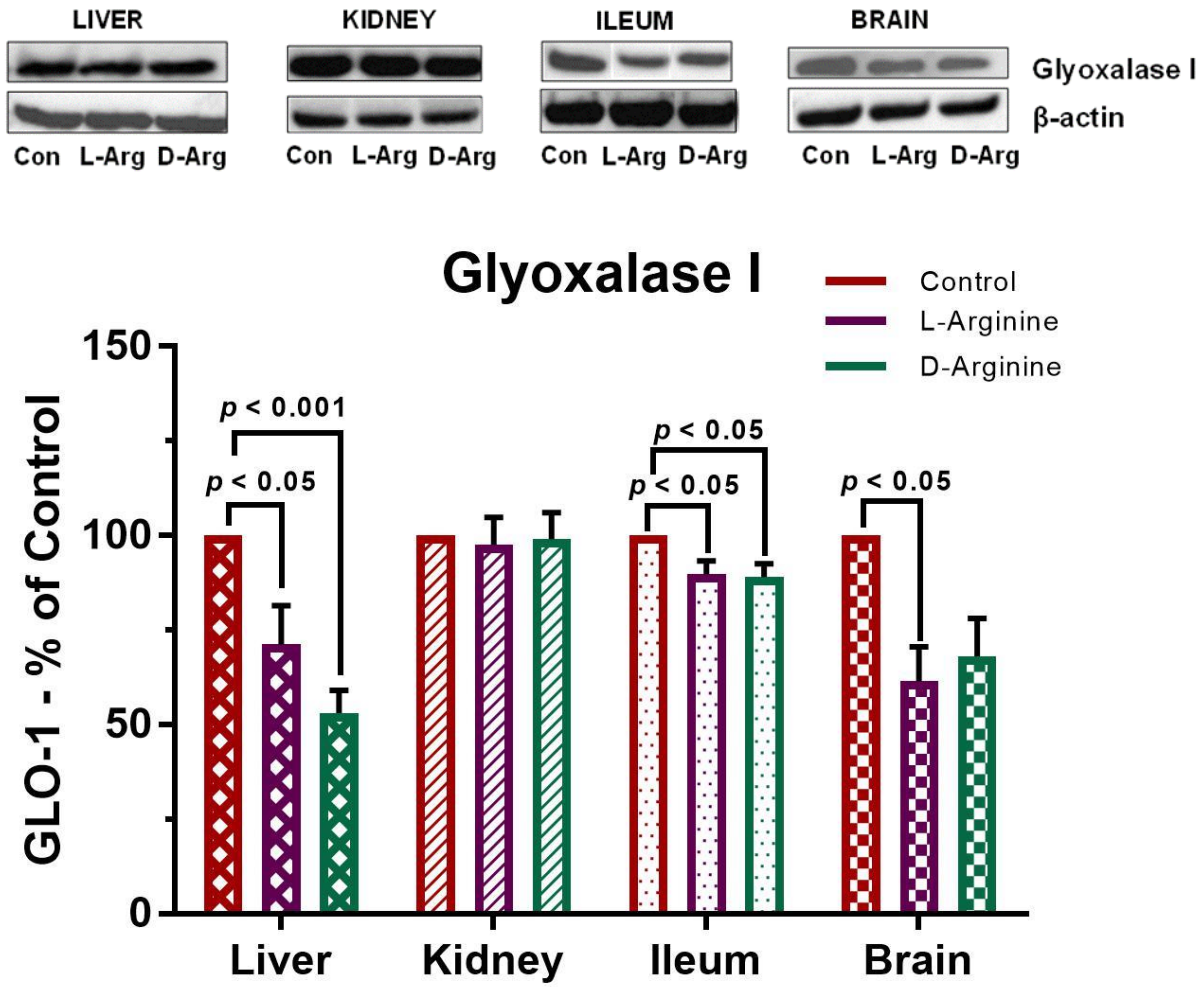

Figure 3.34. Effect of oral arginine (500 mg/kg/day) for 4 weeks on the expression of glyoxalase I in the liver, kidney, ileum and brain of Sprague Dawley rats.

10-week-old male Sprague Dawley rats were treated with L-Arginine (L-Arg) or D-Arginine (D-Arg) in drinking water (500 mg/kg/day) for 4 weeks. Western blotting was used to quantify glyoxalase expression in the liver, kidney, ileum and brain. The blots were quantified using the image analysis program GeneTools from Syngene (Liver, n = 9; Kidney, n = 9; Ileum, n = 11; Brain, n = 3).

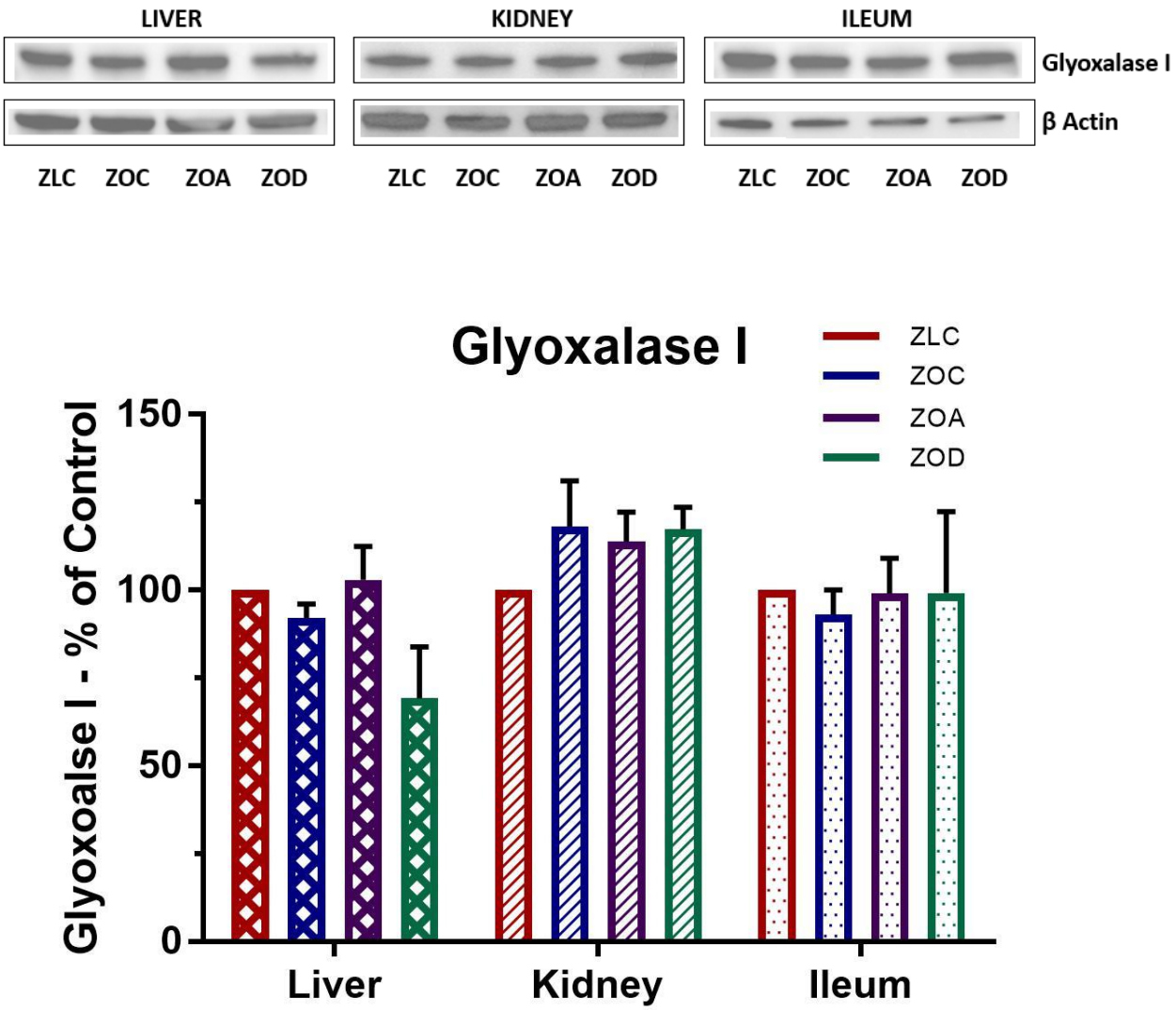

Figure 3.35. Effect of oral arginine (1000 mg/kg/day) for 12 weeks on the expression of glyoxalase I in the liver, kidney, ileum and brain of Zucker rats.

6-week-old male Zucker lean control (ZLC) and Zucker Diabetic Fatty control (ZOC) rats were given normal drinking water for 12 weeks. An additional group of Zucker Diabetic Fatty rats were treated with L-Arginine (ZOA) or D-Arginine (ZOD) in drinking water (1000 mg/kg/day) for 12 weeks. Western blotting was used to quantify arginase I and II expression in the liver, kidney and ileum. The blots were quantified using the image analysis program GeneTools from Syngene (Liver, n = 5-7; Kidney, n = 4-7; Ileum, n = 5-8).

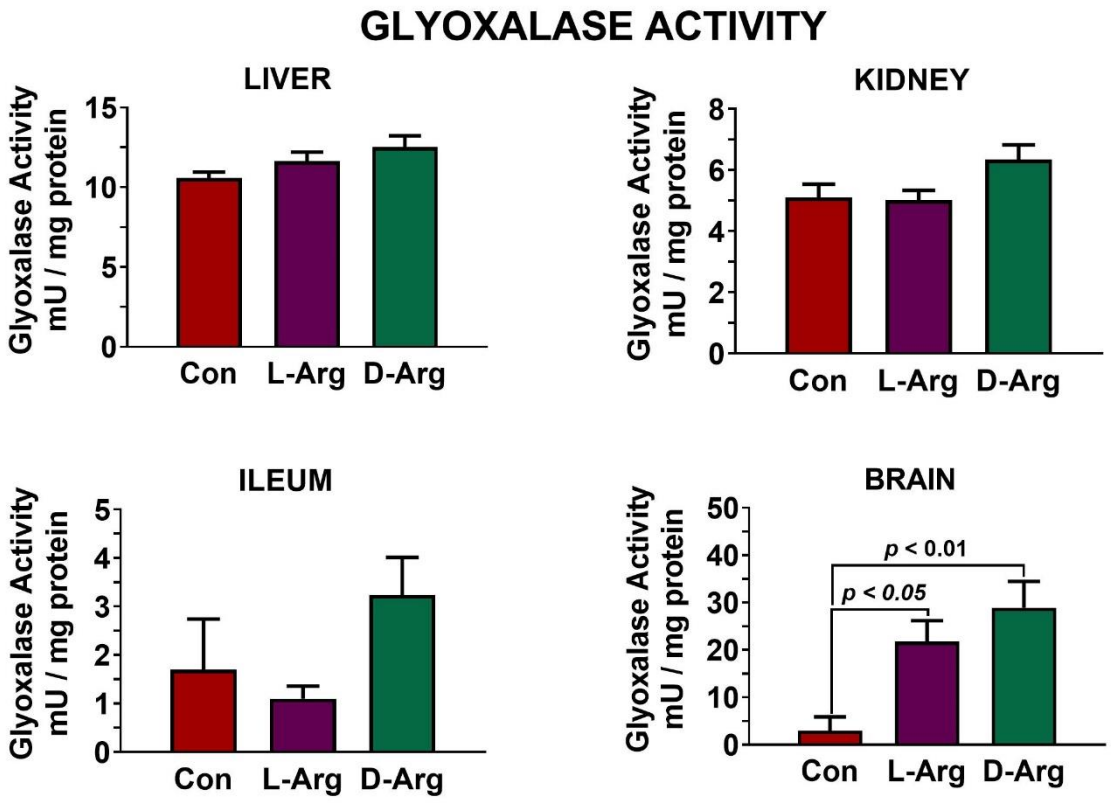

Figure 3.36. Effect of oral arginine (500 mg/kg/day) for 4 weeks on glyoxalase activity in different organs/tissues of Sprague Dawley rats.

10-week-old male Sprague Dawley rats were treated with L-Arginine (L-Arg) or D-Arginine (D-Arg) in drinking water (500 mg/kg/day) for 4 weeks. An assay kit was used to quantify glyoxalase activity (Control, n = 4-7; L-Arg, n = 3-9; D-Arg, n = 7-8).

3.45 Oral L- and D-Arginine supplements (500 mg/kg/day) significantly altered methylglyoxal levels in the kidney, liver, ileum and lungs but not the brain of Sprague Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 4 weeks with 500 mg/kg/day of D-Arg significantly increased MG levels in the kidney as compared to control and the liver as compared to both the control and L-Arg groups (Fig. 3.37). Oral L-Arg supplements of 500 mg/kg/day also significantly increased MG levels in the kidney but did not affect MG levels in the liver as compared to control (Fig. 3.37). In the plasma L-Arg significantly increased MG levels as compared to control and D-Arg (Fig. 3.37). Both L- and D-Arg significantly decreased MG levels in the ileum and lungs as compared to control (Fig. 3.37). Neither L- or D-Arg had any significant effect on MG levels in the brain of Sprague Dawley rats (Fig 3.37).

3.46 D-Arginine supplements (1000 mg/kg/day) for 16 weeks significantly increased MG levels in the plasma of Sprague Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 16 weeks with 1000 mg/kg/day of D-Arg significantly increased plasma levels of MG as compared to both control and L-ARG (Fig 3.38).

3.47 Oral L- and D-Arginine supplements (1000 mg/kg/day) for 12 weeks significantly altered methylglyoxal levels in plasma, lungs, liver and kidney but not ileum or aorta of Zucker rats.

Treatment of 6-week-old male Zucker Diabetic Fatty rats with 1000 mg/kg/day for 12 weeks of L-Arg significantly decreased plasma MG levels as compared to obese controls. (Fig. 3.39). In the lungs L-Arg significantly increased MG levels as compared to both lean and obese controls (Fig. 3.39). Treatment with 1000 mg/kg/day of D-Arg resulted in significantly increased lung, liver and kidney MG levels as compared to the lean control (Fig. 3.39). MG levels in the lungs were also significantly increased by D-Arg as compared to the obese control (Fig. 3.39). In the liver obese controls exhibited significantly increased MG levels as compared to the lean controls, while this increase was also observed in the D-Arg group it did not occur in the L-Arg group (Fig. 3.39).

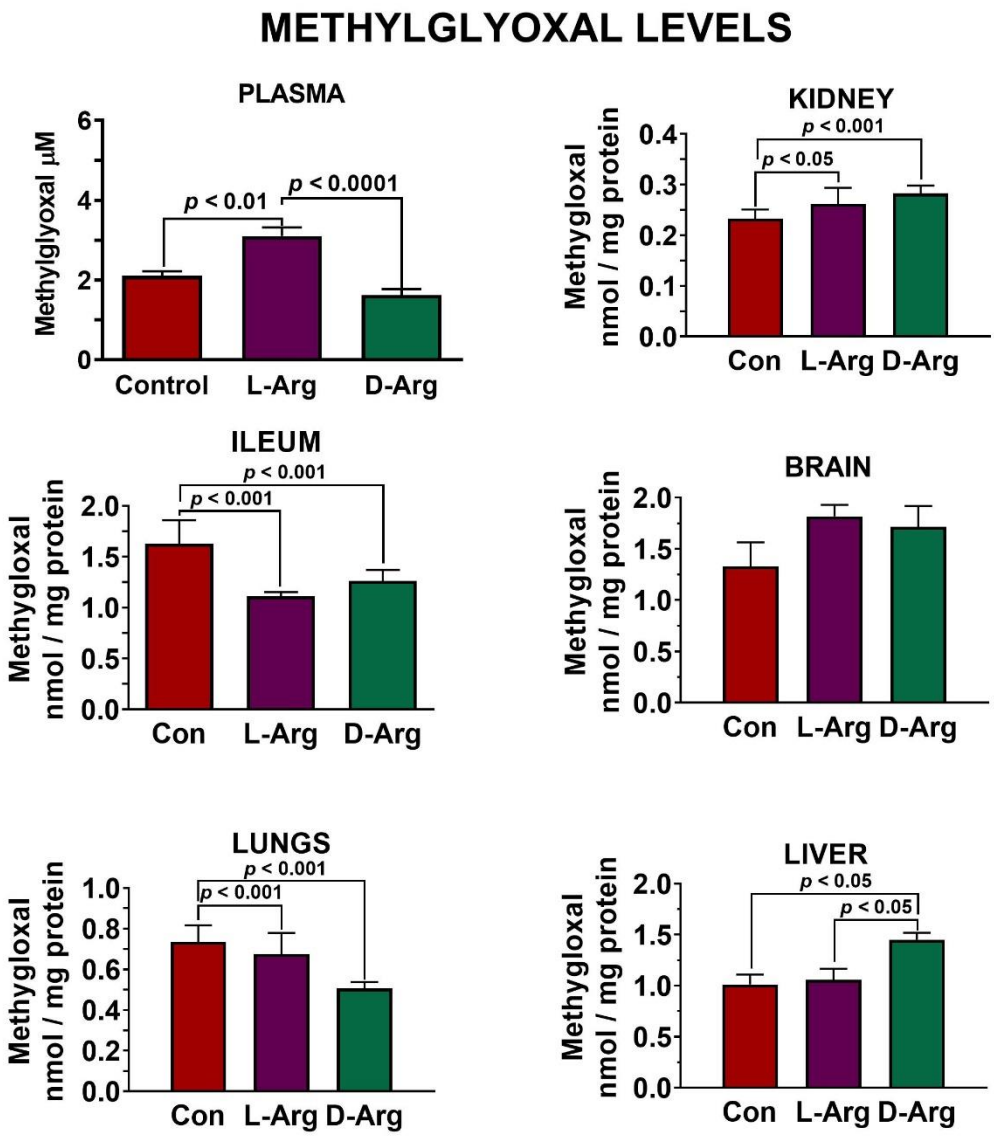

Figure 3.37. Effect of oral arginine (500 mg/kg/day) for 4 weeks on methylglyoxal (a reactive intermediate of mainly glucose metabolism) levels in the plasma and different organs/tissues of Sprague Dawley rats.

10-week-old male Sprague Dawley rats were treated with L-Arginine (L-Arg) or D-Arginine (D-Arg) in drinking water (500 mg/kg/day) for 4 weeks. The organ/tissue was homogenized and MG was derivatized with o-phenylenediamine to form 2-methylquinoxaline which was measured by HPLC (Plasma, *n* = 11-17; Kidney, *n* = 8-9*;* Ileum, *n* = 8; Brain, *n* = 6; Lungs, *n* = 8-9*;* Liver, *n* = 7-9).

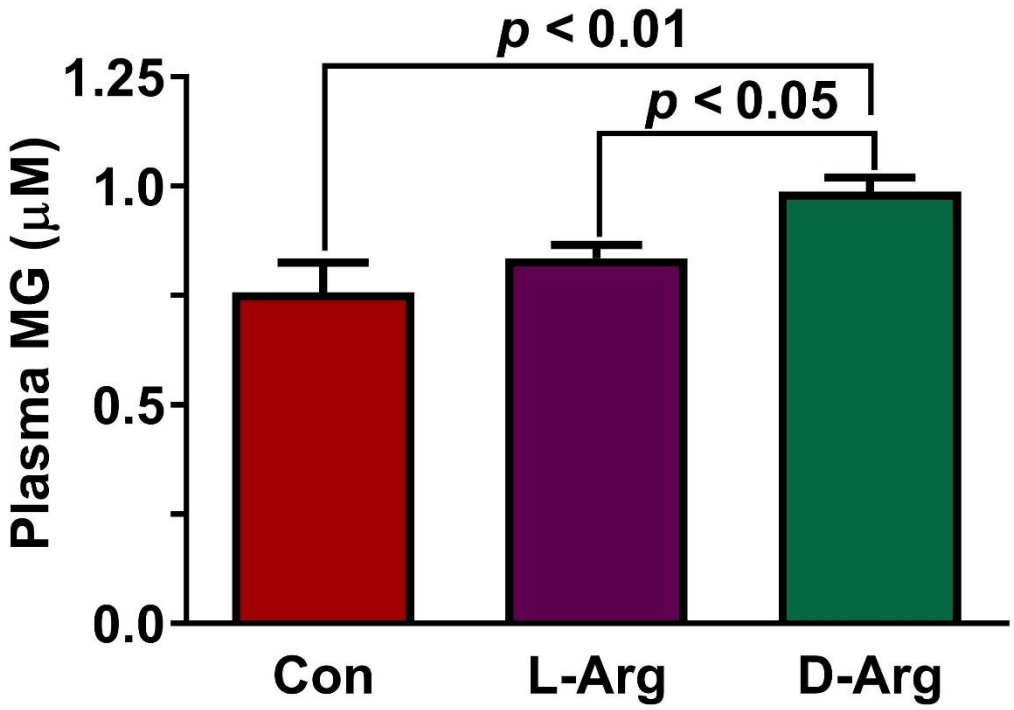

Figure 3.38. Effect of oral arginine (1000 mg/kg/day) for 16 weeks on methylglyoxal (a reactive intermediate of mainly glucose metabolism) levels in the plasma of Sprague Dawley rats.

10-week-old male Sprague Dawley rats were treated with L-Arginine (L-Arg) or D-Arginine (D-Arg) in drinking water (1000 mg/kg/day) for 16 weeks. MG was derivatized with o-phenylenediamine to form 2-methylquinoxaline which was measured by HPLC (Control, $n = 8$; L-Arg, $n = 9$; D-Arg, $n = 8$).

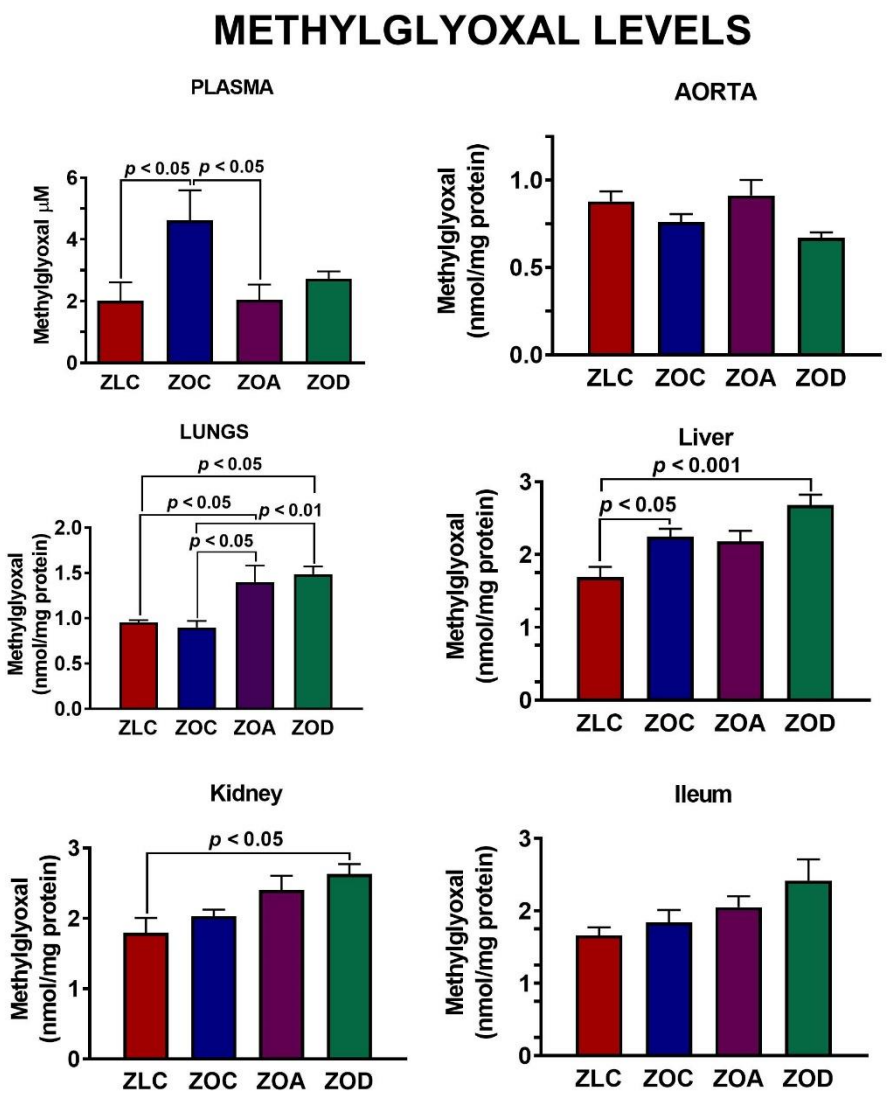

Figure 3.39. Effect of oral arginine (1000 mg/kg/day) for 12 weeks on methylglyoxal (a reactive intermediate of mainly glucose metabolism) levels in the plasma and different organs/tissues of Zucker rats.

6-week-old male Zucker lean control (ZLC) and Zucker Diabetic Fatty control (ZOC) rats were given normal drinking water for 12 weeks. An additional group of Zucker Diabetic Fatty rats were treated with L-Arginine (ZOA) or D-Arginine (ZOD) in drinking water (1000 mg/kg/day) for 12 weeks. The organ/tissue was homogenized and MG was derivatized with o-phenylenediamine to form 2-methylquinoxaline which was measured by HPLC (Plasma, n = 3-5; Aorta, n = 4-5; Lungs, n = 5; Liver, n = 5; Kidney, n = 5; Ileum, n = 5).

3.48 Oral L-Arginine supplements (500 mg/kg/day) significantly increased D-lactate levels in the plasma but decreased D-lactate levels in the brain of Sprague Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 4 weeks with 500 mg/kg/day of L-Arg significantly increased D-lactate levels in the plasma but decreased them in the brain as compared to control (Fig. 3.40). Oppositely, D-lactate levels in the plasma were significantly increased as compared to control (Fig. 3.40). L-Arg supplements had no effect on D-lactate levels in the ileum, liver, kidney or urine of Sprague Dawley rats (Fig. 3.40).

3.49 Oral D-arginine supplements (500 mg/kg/day) significantly increased D-lactate levels in the kidney but did not affect the liver, ileum, kidney, brain or urine of Sprague Dawley rats.

Treatment of 10-week-old male Sprague Dawley rats for 4 weeks with 500 mg/kg/day of D-Arg significantly increased D-lactate levels in the kidney as compared to control (Fig. 3.40). Oral D-Arginine supplements did not significantly alter D-lactate levels in the liver, ileum, kidney, brain or urine (Fig. 3.40).

3.50 Oral D-arginine supplements (1000 mg/kg/day) for 12 weeks significantly increased D-lactate levels in the kidney of Zucker Diabetic Ratty rats.

Treatment of Zucker Diabetic Fatty rats with 1000 mg/kg/day of D-Arg for 12 weeks significantly increased D-lactate levels in the kidney as compared to the lean control and L-Arg group (Fig. 3.41). However, D-Arg did not have any effect on D-lactate levels in the plasma, urine or ileum (Fig. 3.41). L-Arg at the same dose for 12 weeks did not have any significant effect on D-lactate levels in the plasma, urine, ileum or kidney (Fig. 3.41).

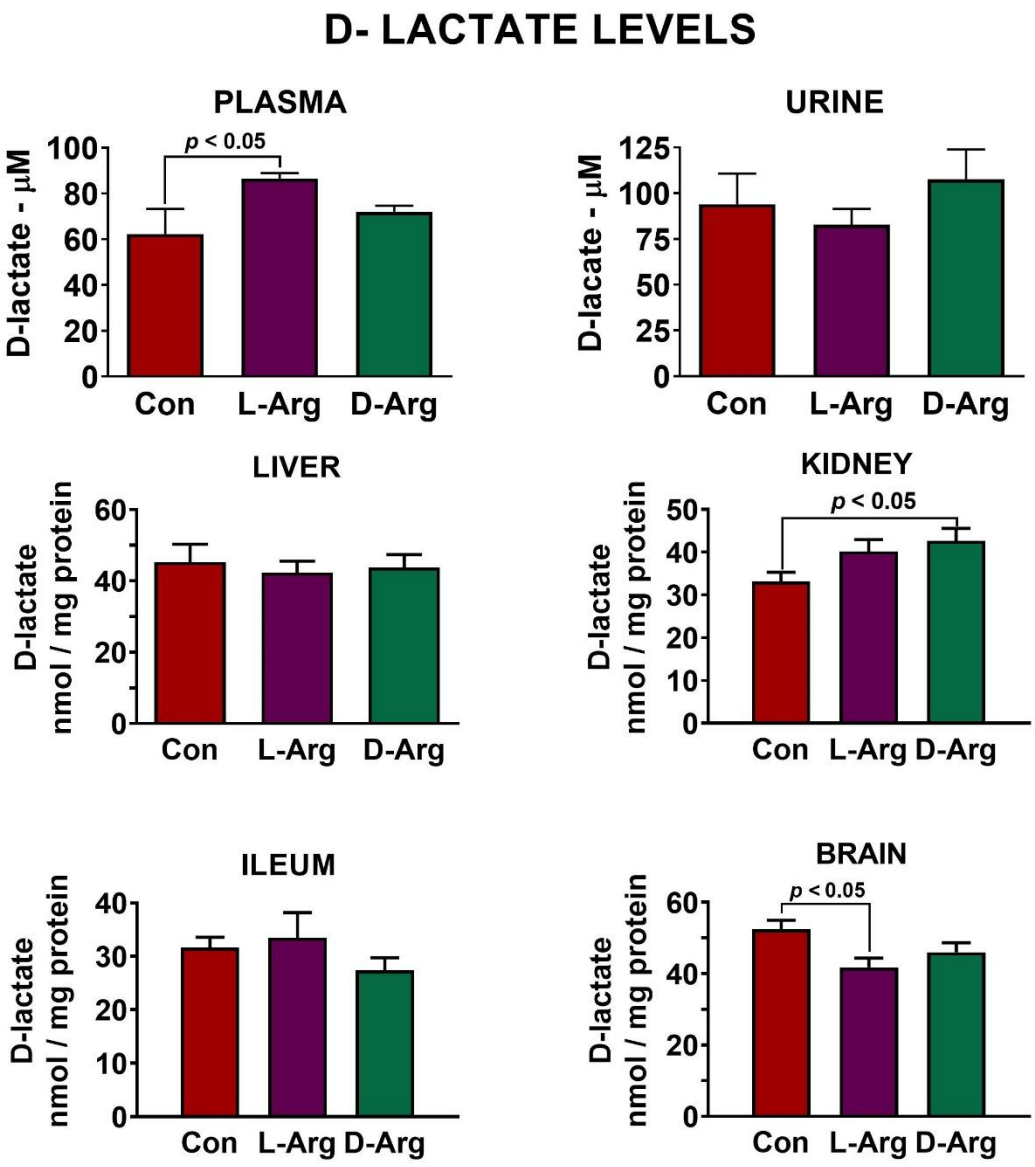

Figure 3.40. Effect of oral arginine (500 mg/kg/day) for 4 weeks on D-lactate (an inert metabolite of methylglyoxal) levels in plasma and different organs/tissues of Sprague Dawley rats.

An assay kit was used to measure NOS activity. 10-week-old male Sprague Dawley rats were treated with L-Arginine (L-Arg) or D-Arginine (D-Arg) in drinking water (500 mg/kg/day) for 4 weeks. An assay kit was used to quantify D-lactate levels (Control, n = 5-7; L-Arg, n = 6-8; D-Arg, n = 5-8).

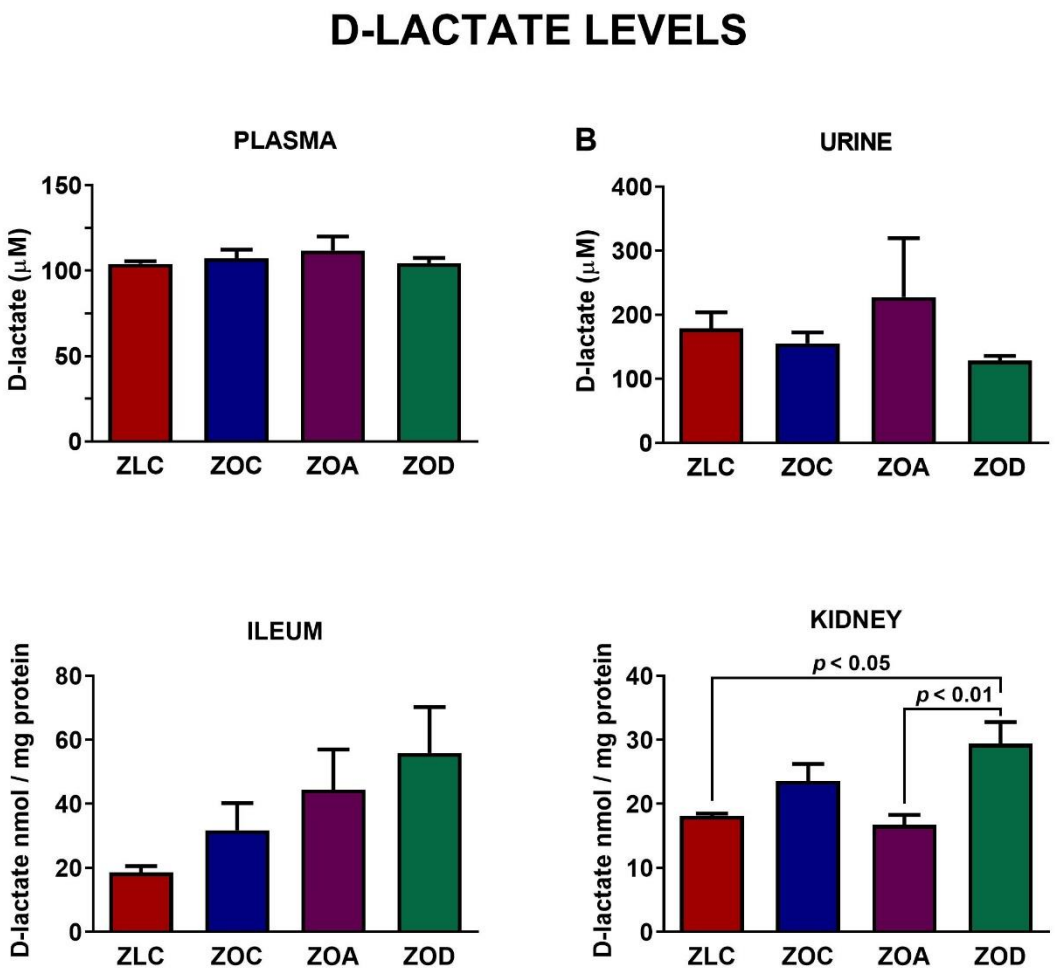

Figure 3.41. Effect of oral arginine (1000 mg/kg/day) for 12 weeks on D-lactate (an inert metabolite of methylglyoxal) levels in plasma and different organs/tissues of Zucker rats.

6-week-old male Zucker lean control (ZLC) and Zucker Diabetic Fatty control (ZOC) rats were given normal drinking water for 12 weeks. An additional group of Zucker Diabetic Fatty rats were treated with L-Arginine (ZOA) or D-Arginine (ZOD) in drinking water (1000 mg/kg/day) for 12 weeks. An assay kit was used to quantify D-lactate levels (ZLC, n = 5; ZOC, n = 4-5; ZOA, n = 5; ZOD, n = 4-5).

3.51 Oral arginine supplements (1000 mg/kg/day) for 12 weeks significantly increased fasting glucose levels in the plasma of Zucker Diabetic Fatty rats.

6-week-old male Zucker Diabetic Fatty control rats given normal drinking water had significantly increased plasma fasting glucose as compared to Zucker lean controls (Fig. 3.42). Treatment of 6-week-old male Zucker Diabetic Fatty rats with 1000 mg/kg/day of L- or D-Arg also significantly increased plasma fasting glucose as compared to Zucker lean controls (Fig. 3.42).

3.52 Oral arginine supplements (1000 mg/kg/day) for 12 weeks did not improved oral glucose tolerance of Zucker Diabetic Fatty rats.

6-week-old male Zucker Diabetic Fatty control rats given normal drinking exhibited a trend towards hindered oral glucose tolerance (glucose – OGTT) or fasting glucose as compared to Zucker lean controls (Fig. 3.42). Treatment of 6-week-old male Zucker Diabetic Fatty rats with 1000 mg/kg/day of L- or D-Arg did not improve oral glucose tolerance or fasting glucose as compared to Zucker lean controls (Fig. 3.42).

3.53 Oral arginine supplements (1000 mg/kg/day) for 12 weeks did not significantly alter fasting insulin levels in the plasma of Zucker Diabetic Fatty rats.

6-week-old male Zucker Diabetic Fatty control rats given normal drinking water did not have significantly different fasting plasma insulin levels as compared to Zucker lean controls (Fig. 3.43). Treatment of 6-week-old male Zucker Diabetic Fatty rats with 1000 mg/kg/day of L- or D-Arg also did not significantly change fasting plasma insulin levels as compared to Zucker lean controls (Fig. 3.43).

3.54 Oral arginine supplements (1000 mg/kg/day) for 12 weeks did not improved glucose mediated insulin responses in Zucker Diabetic Fatty rats.

6-week-old male Zucker Diabetic Fatty control rats given normal drinking showed no significant change in glucose mediated insulin responses (insulin – OGTT) as compared to Zucker lean controls (Fig. 3.43). Treatment of 6-week-old male Zucker Diabetic Fatty rats with 1000 mg/kg/day of L- or D-Arg also did not significantly alter glucose mediated insulin responses as compared to Zucker lean controls (Fig. 3.43).

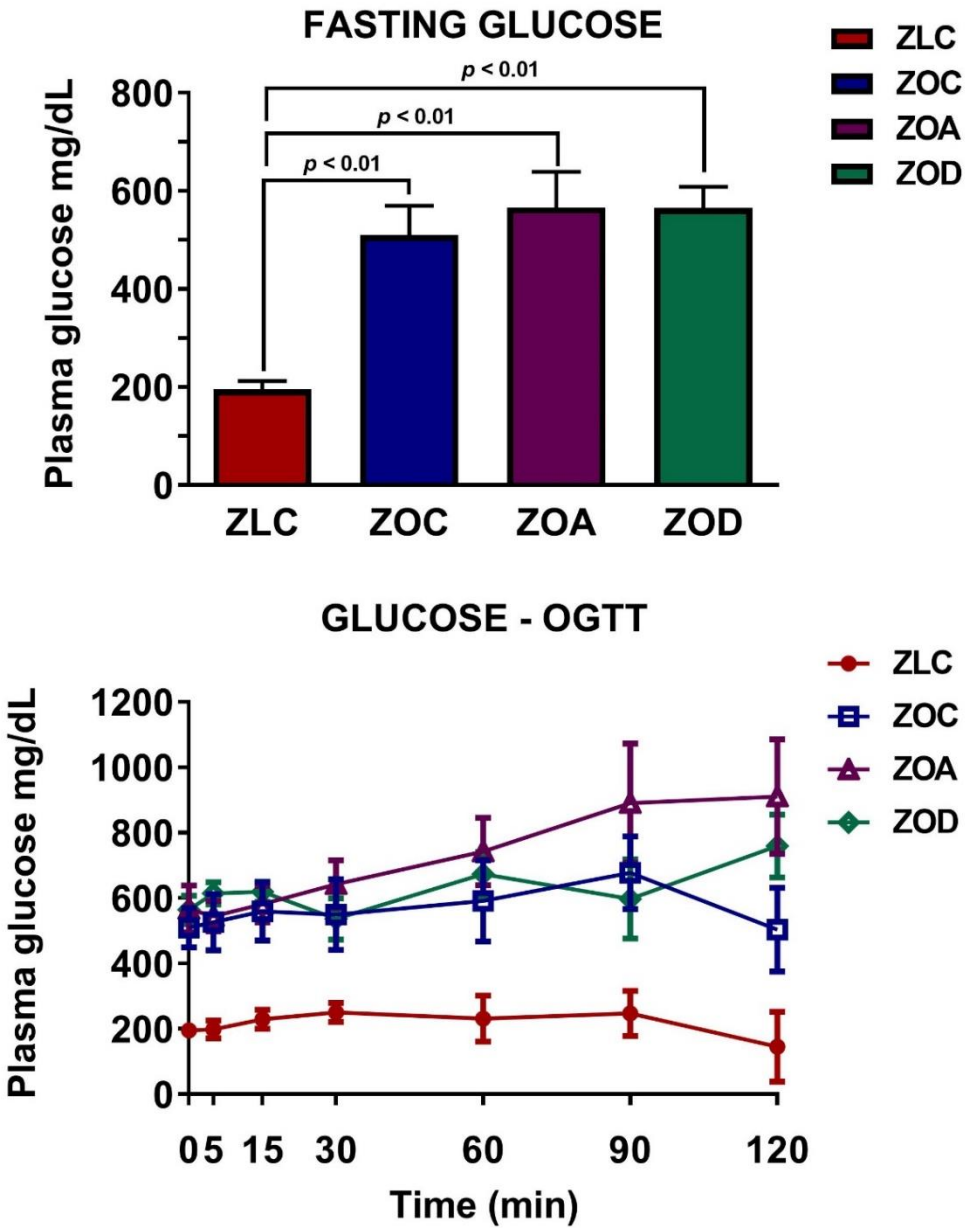

Figure 3.42. Effect of oral arginine (1000 mg/kg/day) for 12 weeks on glucose levels in plasma of Zucker rats.

6-week-old male Zucker lean control (ZLC) and Zucker Diabetic Fatty control (ZOC) rats were given normal drinking water for 12 weeks. An additional group of Zucker Diabetic Fatty rats were treated with L-Arginine (ZOA) or D-Arginine (ZOD) in drinking water (1000 mg/kg/day) for 12 weeks. An assay kit was used to quantify fasting glucose levels in plasma prior to delivering glucose bolus (0 min). Plasma glucose levels in samples taken at 5, 15, 30, 60, 90 and 120 minutes post glucose bolus injection were then analyzed using the same assay kit and plotted to display the oral glucose tolerance test (OGTT). (ZLC, *n* = 2-4; ZOC, *n* = 3-4; ZOA, *n* = 4-5; ZOD, *n* = 3-4).

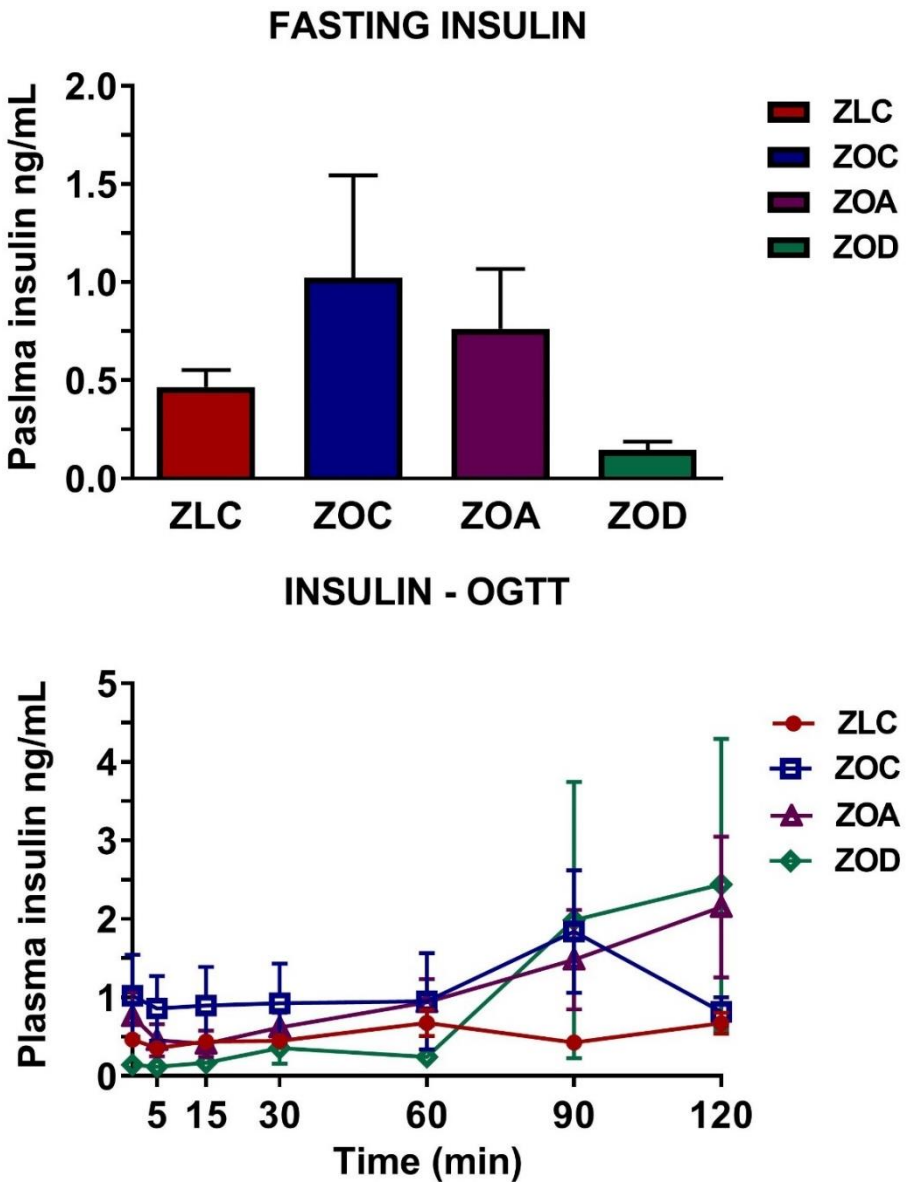

Figure 3.43. Effect of oral arginine (1000 mg/kg/day) for 12 weeks on insulin levels in plasma of Zucker rats.

6-week-old male Zucker lean control (ZLC) and Zucker Diabetic Fatty control (ZOC) rats were given normal drinking water for 12 weeks. An additional group of Zucker Diabetic Fatty rats were treated with L-Arginine (ZOA) or D-Arginine (ZOD) in drinking water (1000 mg/kg/day) for 12 weeks. An assay kit was used to quantify fasting insulin levels in plasma prior to delivering glucose bolus (0 min). Insulin levels were then analyzed in samples taken at 5, 15, 30, 60, 90 and 120 minutes post glucose bolus using the same assay kit to test for insulin responses during the oral glucose tolerance test (OGTT).(ZLC, *n* = 2-4; ZOC, *n* = 3-4; ZOA, *n* = 4-5; ZOD, *n* = 3-4).

3.55 Oral D-Arginine supplements (1000 mg/kg/day) for 12 weeks significantly increased the glucose/insulin ratio in Zucker Diabetic Fatty rats.

6-week-old male Zucker Diabetic Fatty control rats given normal drinking or 1000 mg/kg/day of L-Arg showed no significant change in the glucose/insulin ratio as compared to Zucker lean controls (Fig. 3.44). Treatment of 6-week-old male Zucker Diabetic Fatty rats with 1000 mg/kg/day of D-Arg significantly increased the glucose/insulin ratio as compared to the Zucker lean and Zucker obese controls (Fig. 3.44).

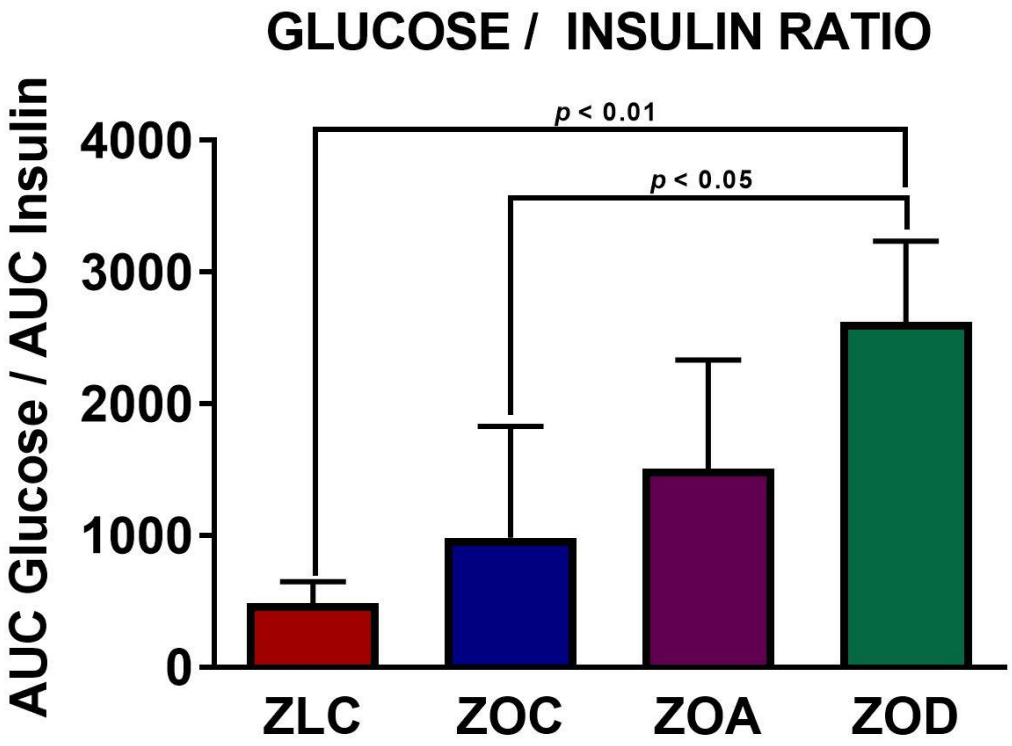

Figure 3.44. Effect of oral arginine (1000 mg/kg/day) for 12 weeks on the plasma glucose/insulin ratio of Zucker lean and Zucker Diabetic Fatty rats.

6-week-old male Zucker lean control (ZLC) and Zucker Diabetic Fatty control (ZOC) rats were given normal drinking water for 12 weeks. An additional group of Zucker Diabetic Fatty rats were treated with L-Arginine (ZOA) or D-Arginine (ZOD) in drinking water (1000 mg/kg/day) for 12 weeks. An assay kit was used to quantify glucose and insulin levels (ZLC, n = 4; ZOC, n = 4; ZOA, n = 4; ZOD, n = 4).

Table 3.1. Summary of results.

Parameter	**500 SD LA**	**500 SD DA**	**1000 SD LA**	**1000 SD DA**	**1000 ZDF ZOC**	**1000 ZDF ZOA**	**1000 ZDF ZOD**
Water Intake	↔	↔	↔	↓ *vs* LA	↑ 18 wks *vs* 6 wks	↑ 18 wks *vs* 6 wks	↑ 18 wks *vs* 6 wks
Body Weight	↔	↔	↔	↔	↔	↔	↔
Arginine Levels	↔	↔	X	X	↔	↔	↔
CAT-1	**↓Li, Il**	↔	X	X	**↓Li, Ao**	**↓Li, Ao, Il**	**↓Li, Ao, Il**
eNOS Pathway							
MAP	↔	↔	↔	↔	↑	**↓ *vs* ZOC**	↔
HR	↔	↔					
EF	X	X	↔	↔	↔	↔	↔
eNOS Expression	**↑Ao, Ki**	**↑Ki**	X	X	**↑Ao; ↓Ki *vs* ZOA**	**↑Ao; ↑Ki *vs*. ZLC, ZOC,ZOD**	**↓Ki *vs* ZOA**
eNOS Activity	↓Lu *vs* DA	↑	X	X	↔	↔	↔
Nitrite Levels	↑Pl *vs* C, DA	↓ *vs* LA	X	X	↓Li	↓Li; ↓ Sk *vs* ZOD	↓Li, ↓Lu, ↑ Sk *vs* ZOA
Arginase Pathway							
Arginase Expression	**↓Il**	**↓Li, Il**	X	X	↑	↑	↑

Arginase Activity	↔	↔	X	X	↔Pl, ↓Il	**↓ Pl vs ZOC; ↓Il**	**↓Il vs ZLC**
Urea Levels	↔	↑Li; ↑Ki *vs* C, LA	X	X	↑Pl	↑Pl	↑Pl

Parameter	**500 SD LA**	**500 SD DA**	**1000 SD LA**	**1000 SD DA**	**1000 ZDF ZOC**	**1000 ZDF ZOA**	**1000 ZDF ZOD**
Arginine Decarboxylase Pathway							
ADC Expression	↑Li	↔	X	X	X	X	X
ADC Activity	↔	↑Il	X	X	X	X	X
Agmatinase Expression	↔	↑Li *vs* LA	X	X	X	X	X
Polyamines	↑Li vs C, DA	↑Pl	X	X	X	X	X
Arginine: glycine amidinotransferase Pathway							
AGAT Expression	↓Li, Ki	↑Li *vs* LA ↑Ki *vs* C, LA	X	X	X	X	X
Creatinine	↓Sk	↑Li	X	X	X	X	X
Glyoxalase Pathway							
Glyoxalase I Expression	↓Li, Il, Br	↓Li, Il	X	X	↔	↔	↔
Gloxalase I Activity	↑Br	↑Br	X	X	X	X	X
MG Levels	↑Ki ↓Il, Lu	↑Ki; ↑Li *vs* LA;	↔	↑ *vs* C, LA	↑Pl *vs* ZLC, ZOA; ↓Lu	↓Pl *vs* ZOC;	↑Lu *vs* ZLC,

		↓Il, Lu			vs ZOA, ZOD; ↑Li	↑Lu vs ZOC	ZOC; ↑Ki; ↑Li
D-lactate Levels	↑Pl ↓Br	↑Ki	X	X	↔	↓ Ki *vs* ZOD	↑ Ki *vs* ZOA,ZOD
Parameter	**500 SD LA**	**500 SD DA**	**1000 SD LA**	**1000 SD DA**	**1000 ZDF ZOC**	**1000 ZDF ZOA**	**1000 ZDF ZOD**
Glucose and Insulin Assays							
Fasting Glucose	X	X	X	X	↑	↑	↑
OGTT Glucose	X	X	X	X	↔	↔	↔
Fasting Insulin	X	X	X	X	↔	↔	↔
OGTT Insulin	X	X	X	X	↔	↔	↔
Glucose/Insulin	X	X	X	X	↔	↔	↑ *vs* ZLC, ZOC

Abbreviations: 500 SD – 500 mg for 4 weeks Sprague Dawley rats; 1000 SD – 1000 mg for 16 weeks Sprague Dawley rats; ADC- arginine decarboxylase; Ao – aorta; Br – brain; C – control; CAT-1 – cationic amino transporter 1; DA – D-Arginine; EF – endothelial function; eNOS – endothelial nitric oxide synthase; AGAT – arginine: glycine amidinotransferase; HR – heart rate; Il – ileum; Ki – kidney; LA – L-Arginine; Li – liver; MAP – mean arterial pressure; OGTT – oral glucose tolerance test; Pl – plasma; Sk – skeletal muscle; wks – weeks; ZLC – Zucker lean control; ZOA – Zucker diabetic fatty rat + L-Arginine; ZOC – Zucker diabetic fatty rat control; ZOD – Zucker diabetic fatty rat + D-Arginine

Symbols: ↔ no change compared to control (Sprague Dawley rats) or Zucker lean control (Zucker diabetic fatty rats); ↑ increase or ↓ decrease compared to control (Sprague Dawley rats) or Zucker lean control (Zucker diabetic fatty rats) unless otherwise stated; X – not assayed.

CHAPTER 4. DISCUSSION

Oral arginine supplements have become more popular following the discovery of its role as a substrate for nitric oxide production and the related cardiovascular benefits. Supplements have been used to treat a wide variety of conditions, however, endpoints in most studies do not involve the direct effect on several enzymes which utilize arginine as a substrate and arginine metabolites (Clarkson et al., 1996; Lekakis et al., 2002; N. Pahlavani et al., 2017). While both L-and D-Arg have proven to be effective *in vitro* scavengers of MG (Dhar et al., 2012), their ability to do so *in vivo* has not been investigated to the best of our knowledge. Additionally, most studies only investigate the effect of L-Arg, but not D-Arg, as the latter is thought to be metabolically inert in mammals (Morris et al., 1997; Palmer et al., 1988). Thus, we sought to investigate if orally administered L-Arg but not D-Arg in SD and ZDF rats could alter the expression/activity of arginine related enzymes and metabolites in addition to lowering basal levels of MG. We also wanted to investigate if arginine supplements were more effective in a pathological model *vs* a physiological model by assessing the effect on MAP and endothelial function in both ZDF and SD rats. Finally, as MG and MG derived AGEs are increased in T2DM (Kong et al., 2014) we also wanted to explore the effects of arginine supplements on glucose tolerance and insulin responses.

Treatment of SD rats with 500 mg/kg/day of L-or D-Arg did not result in any mortalities. Similarly, increasing the dose to 1000 mg/kg/day did not result in any mortalities of SD rats. As discussed in more detail below, some of the L-Arg (1000 mg) ZDF rats were lost but all deaths were due to complications during the cannulation procedure and not related to the arginine itself. In the ZDF D-Arg (1000 mg) group one animal had to be euthanized at the end of the treatment period due to immobility of the hind limbs. As this is not a symptom that has ever been described in relation to arginine supplements and was an isolated incident, we do not believe this was a result of the D-Arg supplements.

As mentioned in the methods delivering high doses were a concern as very high intraperitoneal (i.p.) doses of 500 mg/100 g of body weight has been shown to elicit pancreatitis in rats (Heygi et al., 2004). An important point to note here is that high doses of arginine have to be administered parenterally (i.p. or i.v.) in order to induce pancreatitis. Thus, in our study at our highest dose of 1000 mg/kg/day with an average body weight of 500 g rats were receiving 500 mg a day gradually through the oral route as compared to 2500 mg delivered i.p. in the study mentioned (Heygi et al., 2004). Thus, our dose is highly unlikely to cause pancreatitis not only because it is

5x lower but it is also delivered orally thus decreasing the bioavailability of the dose. For example, with an approximate bioavailability of 60% only 300 mg was reaching the bloodstream as compared to 2500 mg. Of greater concern would have been the effect of osmotic stress on the animals as arginine was delivered as a salt. Mammals have a extracellular fluid osmotic set point of 300 mosmol kg-1 (Bourque, 2008). With arginine having a molecular weight of 174 and animals in our study having an average water intake of 100 mL per day and body weight of 500 g we can estimate the molarity of their drinking water at the 1000 mg dose to be ~ 0.029 mol/L. With 1 mosmol kg-1 being equal to 1 mmol/L then the rat's drinking water was ~ 29 mosmol kg-1. Thus, osmotic stress should not have occurred as the drinking water was still hypotonic as compared to rat's plasma. This absence in osmotic stress is evidenced by the lack of increased blood pressure in any arginine treatment groups as compared to control, and also as evidenced by no loss of body weight (Fig. 3.11, 3.12, 3.13).

Treatment of SD rats with 500 mg/kg/day of L-or D-Arg did not significantly alter water intake (Fig. 3.1). Additionally, treatment of SD rats with 1000 mg/kg/day for 16 weeks of D-Arg, but not L-Arg, did significantly decrease water intake as compared to the L-Arg group (Fig. 3.2). D-Arg is a yellow powder, which unlike L-Arg, alters the color of the water and has a distinct smell. It is possible that the altered color and smell at the higher dose resulted in decreased water intake. However, as the amount of arginine added to the water bottle was adjusted based on daily water intake, this reduction in the D-Arg group would not have altered the dose being received. In ZDF rats, water intake was significantly increased in the ZOC, ZOA and ZOD groups at 18 weeks as compared to each group's respective daily water intake at 6 weeks (Fig. 3.3). This increase at 18 weeks *vs* 6 weeks was not observed in the ZLC group (Fig. 3.3). Additionally, at 18 weeks the ZOC, ZOA and ZOD groups were consuming significantly more water than the ZLC group (Fig. 3.3). This increase in water intake was a result of the ZOC, ZOA and ZOD groups being diabetic. As the ZOC group had significantly increased water intake to similar levels as the arginine supplemented groups we can conclude that the increase is due to polydipsia or excessive thirst, one of the classic symptoms of untreated diabetes. Increased thirst is a result of excess water loss in the urine, when kidneys can no longer handle high glucose levels the glucose is excreted in the urine along with water resulting in dehydration (Kumar et al., 2012).

The body weight in SD rats was also unchanged by both the low and high dose of L- and D-Arg compared to the control group (Fig. 3.4 and 3.5). This is in agreement with a study that found

that while oral L-Arg supplements significantly decreased white adipose tissue, they did not significantly alter overall body weight (W. Jobgen, Fu, et al., 2009). Similarly, treatment of 6-week-old ZDF rats with 1000 mg/kg/day of L- or D-Arg did not significantly alter body weight at 18 weeks as compared to the obese or lean controls (Fig. 3.6).

Treatment of 10-week-old male SD rats with 500 mg/kg/day for 4 weeks of oral L- or D-Arg did not significantly affect the plasma or tissue/organs levels of arginine (Fig. 3.7). Similarly, treatment of 6-week-old male ZDF rats with 1000 mg/kg/day of L-or D-Arg did not significantly alter plasma or tissue/organ arginine levels (Fig. 3.8). This was not a surprising result as approximately 40% of oral arginine is metabolized by highly active intestinal arginase enzymes before reaching the bloodstream (Castillo, Chapman, Sanchez, et al., 1993). Additionally, other studies investigating the distribution and half-life of arginine found that they could not detect any changes in arginine levels more than two (Noeh et al., 1996) and four hours (Guoyao Wu et al., 2007) after the dose was given. As we do not collect plasma or harvest tissues/organs within this time frame, a lack of altered arginine levels was expected. Performing a pharmacokinetic study using a single acute dose of arginine given *via* i.v and oral gavage followed by immediate plasma and tissue/organ collection would most likely result in detectable changes in arginine levels.

Interestingly, expression of CAT-1 was significantly decreased by 500 mg of L-Arg in the ileum and liver of SD rats as compared to control (Fig. 3.9). In ZDF rats, both L- and D-Arg (1000 mg) significantly decreased CAT-1 expression in the liver, ileum and aorta as compared to lean controls (Fig. 3.10). CAT-1 levels in the ileum were assayed as CAT-1 is the transporter required to absorb oral arginine into the blood stream (White, 1985). The liver was also assayed as this is the second site after the ileum effected by oral arginine loading. Finally, CAT-1 in the aorta is known to colocalize in in the caveolae of the endothelial cell with eNOS (S. Shin et al., 2011). As CAT-1 plays a role in arginine absorption from the intestines (Closs et al., 2004), its decreased expression in the ileum may have been in part why arginine levels remained unchanged despite supplementation. Additionally, downregulation of the transporter in response to L-Arg may be an adaptation to help combat increased oral arginine. The downregulation in response to D-Arg however is extremely puzzling and novel. Interestingly, these results also support a study in which arginine deficiency resulted in the opposite effect, with a significant upregulation of CAT-1 expression and mRNA being observed (James Fernandez et al., 2003). Finally, this alteration in CAT-1 expression can be problematic long term as it is a non-specific transporter required for the

absorption of other biomolecules from the intestines such as the essential amino acid lysine. In line with this fact, animal studies have shown that arginine supplementation results in decreased levels of lysine in the plasma (Anderson, 1984; Holecek & Sispera, 2016).

Oral arginine supplements are most often taken with the intent of affecting the NOS-NO pathway. When we investigated the effect of oral arginine supplements on MAP, we found that neither 500 mg/kg/day or 1000 mg/kg/day L- or D-Arg were able to lower MAP in SD rats (Fig. 3.11 and 3.12). In ZDF rats, obese controls had significantly increased MAP as compared to lean controls. This effect was anticipated given that T2DM has negative effects on the vasculature such as increased oxidative stress and arginase activity both of which result in endothelial dysfunction *via* decreased NO (Dhar et al., 2012). Interestingly, 1000 mg/kg/day of L-Arg, but not D-Arg, for 12 weeks was able to significantly lower MAP as compared to obese controls to levels even slightly lower than the lean controls (Fig. 3.13). This is in line with current trend in literature whereby L-Arg supplements only appear to lower blood pressure in disease states such as T2DM (Lucotti et al., 2006), hypertension (Lekakis et al., 2002) and hypercholesterolemia (Clarkson et al., 1996) but not healthy individuals (Ast et al., 2011; Naseh Pahlavani et al., 2014). We also found similar results when assessing endothelial function. In SD rats given 1000 mg/kg/day of L- or D-Arg, we did not observe any significant improvement in endothelial dependent relaxation in response to various doses of ACh (Fig. 3.14). In the ZDF rats given the same dose we found a trend towards improved ability to vasodilate in response to L-Arg as compared to both the obese and lean controls (Fig. 3.15). This improvement in respect to obese controls would have most likely been significant if we had a higher sample size. Unfortunately, one animal we could not successfully cannulate the vein so blood pressure was recorded but not hypotensive responses, another animal passed away during the procedure from blood loss and the file of a third animal was not saved resulting in a sample size of two for the L-Arg group. Heart rate was not altered by either isomer or dose in both SD and ZDF rats (Fig. 3.11, 3.12 and 3.12).

eNOS expression in the aorta and kidney of SD rats was significantly increased by L-Arg (500 mg) which may have been an adaptive response to increased substrate availability (Fig. 3.16). Oddly, D-Arg (500) also significantly increased eNOS expression in the kidney (Fig. 3.16). At this time no good explanation can be made as to why this occurred especially as D-Arg is not thought to be a substrate of eNOS (Palmer et al., 1988). The activity of NOS in various tissues/organs of SD rats (Fig. 3.18) was not affected by L-Arg (500 mg). This may explain why MAP was not

decreased (Fig. 3.11) in SD rats despite increased eNOS expression. Other studies have also previously reported unchanged eNOS activity despite L-Arg supplementation (Álvares et al., 2011; Bennett-Richards et al., 2002). Oddly, NOS activity was significantly increased in the lungs of SD rats by D-Arg (500 mg) as compared to both the control and L-Arg groups (Fig. 3.18). In ZDF rats both L- and D-Arg (1000 mg) significantly increased eNOS expression in the aorta as compared to lean control (Fig. 3.17). Interestingly, the obese controls also had significantly increased eNOS expression as compared to lean controls (Fig. 3.17). Perhaps this upregulation was an attempt to overcome diabetes induced hypertension. As observed with the SD rats, expression levels of eNOS in the aorta does not appear to match alterations in MAP (Fig. 3.11). In the kidney of ZDF rats L-Arg (1000 mg) significantly increased eNOS expression as compared to lean controls, obese controls and D-Arg. Potentially, increased vasodilation in renal vasculature allows for enhanced glomerular filtration rates and water excretion leading to the decreased MAP observed in the ZOA group (Fig. 3.13). However, despite increases in eNOS expression, the activity of NOS enzymes was unchanged by L- or D-Arg (1000 mg) in all assayed organs of ZDF rats (Fig. 3.19). L-Arg (500 mg) significantly increased plasma NOx levels in SD rats as compared to control and D-Arg (Fig. 3.20). None of the assayed organs including the aorta displayed any significant changes in NOx levels following treatment with L-Arg. The increase in plasma NOx may be explained by the arginine paradox (S. Shin et al., 2011) which essentially states that caveolar eNOS in endothelial cells has easier access to plasma arginine due to co-localization with CAT-1 (S. Shin et al., 2011). If most of the NO produced by eNOS is diffusing from the caveoli into the plasma rather than onto the smooth muscle this may also explain unchanged mean arterial pressure in SD rats despite increased plasma NOx levels (Fig. 3.20). Finally, L-Arg (1000 mg) supplementation resulted in decreased skeletal muscle NOx levels as compared to D-Arg (1000 mg, Fig. 3.21), an effect which was not observed in SD rats. D-Arg (1000 mg) surprisingly decreased NOx levels in the liver and lungs (Fig. 3.21). The ZOC group also had significantly decreased kidney NOx levels as compared to the ZLC group (Fig. 3.21). These results may have been explained by increased arginase activity; however, arginase activity was unaffected in these organs (Fig. 3.25).

The arginase pathway tends to work in opposition to eNOS. Not only does it steal L-Arg from eNOS as it has a V_{max} ~1000 times greater (Guoyao Wu & Morris, 1998), but it also opposes many of the vasoprotective actions of eNOS. For example, by stealing L-Arg, arginase decreases NO production and thus vasodilation. Arginase also increases endothelial and vascular smooth muscle

proliferation leading to the thickening of blood vessels. Finally, arginases increase vascular collagen synthesis and fibrosis which can lead to the stiffening of vascular walls (Durante et al., 2007). Treatment of SD rats with L-Arg (500 mg) significantly decreased arginase II expression in the ileum while D-Arg significantly decreased arginase II expression in the ileum and arginase I in the liver (Fig. 3.22). Neither isomer had any significant effect on arginase II expression in the kidney of SD rats (Fig 3.22). The reduction observed in the ileum of SD rats by L-Arg (500 mg) may be caused by excess arginine being delivered to the intestines *via* oral supplementation. Especially as the intestines are known to have very high arginase II activity as compared to other organs and is the first organ affected by dietary arginine loading (Guoyao Wu et al., 2007). The effect of D-Arg (500 mg) on arginase II expression in the ileum and arginase I in the liver is unexpected and as with its effect on other enzymes that utilize L-Arg will require further studies to investigate if it potentially competes with endogenous L-Arg for enzyme binding. However, this theory is currently one we treat with skepticism until further studies can be completed. The lack of effect observed in the kidney may be due to not enough arginine reaching the kidney as it is metabolized so quickly before it reaches the renal system.

In ZDF rats neither L- or D-Arg (1000 mg) had a significant effect on arginase I expression in the liver or arginase II expression in the kidney (Fig. 3.23). In the ileum arginase II expression was significantly increased in the Zucker obese controls as compared to the lean controls (Fig. 3.23). This is not surprising as arginase is known to increase in disease states including T2DM, hypertension and obesity (Romero et al., 2008). Oddly, in opposition to the SD rats both L-and D-Arg (1000 mg) significantly increased arginase expression in the ileum (Fig. 3.23). It has been reported that exogenous arginine supplementation can increase in arginase expression (Dioguardi, 2011) which may be the case here. It is odd that the opposite was observed in the ileum of SD rats but this may be explained by the fact that arginase expression is already heightened in the disease state *i.e.* Zucker obese controls and thus the ability of arginine supplements to attenuate this may be hindered.

Previous studies in which Wistar rats were chronically administered L-Arg reported decreased arginase activity (Moretto et al., 2015). We did not observe this decrease in arginase activity when SD rats were supplemented with L-Arg (500 mg). Similarly, in SD rats, D-Arg (500 mg) did not have any effect on arginase activity in the plasma or any of the organs assayed (Fig. 3.24). As expression and activity of enzymes are often not directly proportional it is not surprising that while

arginase I/II expression was altered activity was not. In ZDF rats, obese controls had increased plasma arginase activity as compared to the lean controls. L-Arg (1000 mg) was able to attenuate increased plasma arginase activity resulting in levels significantly lower than obese controls and even below lean controls (Fig. 3.25). Interestingly, arginase activity in the ileum was significantly decreased in the ZOC, ZOA and ZOD groups as compared to the lean controls (Fig. 3.25). This decrease in arginase activity is in opposition to the increased arginase expression (Fig. 3.23) observed in the ileum of these three groups. The products of arginase, urea and L-ornithine, are known to exert negative feedback inhibition on arginase (Hunter A, Downs, 1945). It is possible that increased urea as observed in the plasma of ZOA (Fig. 3.27) rats is responsible for decreased arginase activity. Urea levels in the ileum were not assayed but it would be interesting to investigate if heightened levels of urea or L-ornithine in the ileum are the cause of decreased arginase activity. Alternatively, the decrease in arginase activity may simply be a direct result of exogenous arginine as observed in the study by Moretto *et al.* (2015). Finally, this decrease in arginase activity may help explain why MAP was decreased in ZDF but not SD rats. Arginase enzymes are known to be highly active in the ileum and greatly decrease the bioavailability of oral arginine supplements (Guoyao Wu et al., 2007). Thus, lower arginase activity in the intestine may result in higher bioavailability and thus increased efficacy.

Urea production in the plasma, urine and organs including the lungs, brain and skeletal muscle of SD rats were not affected by either L- or D-Arg (500 mg). Interestingly, D-Arg (500 mg) did increase urea in the liver and kidney (Fig. 3.26). Again, this was a surprising and unexpected result that could indicate potential adverse effects. Urea production in ZDF rats was significantly increased in the plasma of the ZOC, ZOA and ZOD groups (Fig. 3.27). As the ZOC group had increased plasma urea levels similar to those seen in the ZOA and ZOD group, this effect may be due to the obese and diabetic state of the rats and not the arginine supplements.

The ADC pathway shares common metabolites with the arginase pathway (S M Morris, 2004). ADC metabolizes L-Arg into agmatine which is then metabolized into urea and L-ornithine, a precursor for polyamines. The ADC pathway is known to be an alternative pathway for producing polyamines when the arginase pathway is not functioning properly or cannot keep up with increased demands such as during rapid growth or pregnancy (Xiaoqiu Wang et al., 2014). In SD rats given L-Arg (500 mg) ADC expression was significantly increased in the liver but not the brain (Fig. 3.28). This may possibly have been an adaptive response to the first pass of orally

absorbed arginine in the liver. Another possibility may be the ADC pathway making up for the decreased arginase expression in the ileum (Fig. 3.22) which resulted from L-Arg supplementation. D-Arg (500 mg) did not affect ADC expression, but did significantly increase the enzyme's concentration in the ileum (Fig. 3.29). Again, this was unexpected as mammals are not known to utilize D-Arg as a substrate. D-Arg also significantly decreased agmatinase expression in the liver as compared to L-Arg (Fig. 3.30).

L-Arg did not significantly alter agmatinase expression in any of the organs assayed (Fig. 3.30). We had attempted multiple times to assay agmatine levels *via* HPLC but were unable to reproducibly do so. We mainly struggled with finding a protocol detailed enough to allow us to properly repeat. For example, while many protocols gave the derivatizing agent and mobile phase used, they often did not provide the concentrations of reagents required to replicate each solution. We often tried to guess and fill in the blanks ourselves which lead to results that did not seem accurate. For example, we often would have standard curves that did not makes sense e.g. a standard with less agmatine would produce a larger area under the curve than a standard with more agmatine. We did however measure total polyamine levels and found them to be significantly increased in the liver (Fig. 3.31) by L-Arg (500 mg). This increase can be explained by the increased expression of ADC in the liver following L-Arg (500 mg) supplementation. Finally, plasma polyamine levels are significantly increased by D-Arg (500 mg, Fig 3.31). The reason for the increase is difficult to explain, but the enhanced plasma levels by D-Arg may explain the decrease in polyamine levels as compared to the L-Arg group in the liver. Polyamines are known to negatively regulate their production through the an antizyme which inhibits the rate limiting enzyme for polyamine production, ornithine decarboxylase (Satriano et al., 1998).

The final arginine metabolic pathway, the AGAT pathway, is responsible for producing Cr from L-Arg. When SD rats were supplemented with L-Arg (500 mg) we observed a significant decrease in AGAT expression in the liver and kidney (Fig. 3.32). However, neither of these organs experienced any alterations in Cr levels (Fig. 3.33). AGAT has highest activity in the kidney, pancreas and brain. The second enzyme in this pathway, GATM, converts guanidinoacetate to creatine. Activity of GATM is highest in the liver, pancreas and kidney (Van Pilsum et al., 1972). It would be interesting to measure activity/expression of GATM to see if maybe down- or upregulation of this enzyme is contributing to unaltered creatinine levels in the liver and kidney. It

may also be beneficial to quantify guanidinoacetate levels in the liver and kidney to create a more complete understanding of how L-Arg affects this pathway (Fig. 3.33).

We were not able to find assays to directly measure creatine levels, so instead we ended up measuring creatinine levels. As discussed in the introduction, since most of the creatinine is derived from creatine, and in the absence of renal dysfunction and changes in muscle mass or creatine intake, we hoped that creatinine levels would more or less reflect creatine levels in our measurements. L-Arg (500 mg) in SD rats did significantly decrease creatinine levels in the skeletal muscle. Approximately 95% of Cr is found in the skeletal muscle where it serves as a transient energy source (Kreider et al., 2017). Decreased creatinine levels in the skeletal muscle could indicate skeletal muscle loss as creatinine levels and excretion are used as a rough estimate of total muscle mass (Wyss & Kaddurah-Daouk, 2000). In SD rats D-Arg (500 mg) significantly decreased AGAT expression in the liver but increased expression in the kidney (Fig. 3.32). Again, it is puzzling that D-Arg can alter expression of enzymes which are not believed to utilize this isomer as a substrate. In the liver we observed a significant increase in creatinine due to D-Arg supplementation (Fig. 3.32) despite the trend towards a decrease in AGAT expression (Fig. 3.33). This may be due to the theory proposed by Brosnan & Brosnan (2004) in which guanidinoacetate produced in the kidney by AGAT is released into the bloodstream where it then travels to the liver and is converted to Cr by GATM. Thus, as mentioned above, assaying GATM may help us have a better understanding of how Cr levels are affected by arginine supplements. Oppositely, the decrease in AGAT expression in the liver by D-Arg may be due to the increased Cr levels as high Cr levels are known to downregulate AGAT mRNA expression (Van Pilsum, Stephens, & Taylor, 1972).

As discussed previously we wanted to understand the ability of oral arginine supplements to scavenge MG *in vivo*. To do so, we sought to observe the effect of oral arginine supplements on basal MG levels as well as the metabolic pathway responsible for detoxifying MG – the glyoxalase system. In SD rats oral L- and D-Arg supplements (500 mg) significantly decreased glyoxalase I expression in the liver and the ileum (Fig. 3.34). Expression was also significantly decreased in the brain by L-Arg (500 mg, Fig. 3.34). Glyoxalase I activity was unaffected by L-and D-Arg (500 mg) in all organs except the brain where activity was significantly increased (Fig. 3.36). In the ileum and lung of SD rats MG was significantly decreased by both L- and D-Arg (500 mg). Oppositely, both L-and D-Arg (500 mg) significantly increased MG levels in the kidney of SD rats

(Fig. 3.37). MG levels were also significantly increased by D-Arg (500 mg) in the liver (Fig. 3.37). Plasma MG levels were significantly increased by L-Arg (500 mg, Fig. 3.37) but remained at control levels when the dose was increased to 1000 mg (Fig. 3.38). This may suggest that a higher dose is more effective at scavenging MG. The product of MG detoxification, D-lactate was relatively unchanged in most organs of arginine supplemented SD rats. L-Arg (500 mg) resulted in significantly increased plasma D-lactate but decreased brain D-lactate (Fig. 3.40). D-Arg (500 mg) only affected D-lactate levels in the kidney which were significantly increased (Fig. 3.40).

As the ileum presumably receives the greatest proportion of oral arginine, it makes sense that MG levels are decreased (Fig. 3.36) in the presence of a known *in vitro* scavenger. Binding of MG *via* L- and D-Arg would also explain the significant decrease in glyoxalase I expression in the ileum (Fig. 3.34). For example, if arginine is binding and detoxifying MG, then there is less of a need for the glyoxalase system resulting in downregulation. One would then expect lower levels of D-lactate in the ileum, however, no significant changes were observed (Fig. 3.40). This may be explained by the fact that gastrointestinal bacteria produce and utilize D-lactate (Ewaschuk et al., 2005), thus, in this organ particularly it is hard to link D-lactate levels to MG metabolism. In the liver of SD rats, decreased glyoxalase I expression (Fig. 3.34) did not result in altered D-lactate levels (Fig. 3.40). This may be explained by a few mechanisms. The first being that MG can also be metabolized into L-lactate (Chakraborty, Karmakar, & Chakravortty, 2014). Thus, it may have been more effective to assay organs for both D- and L-lactate. Additionally, studies have shown that D-lactate produced *via* the glyoxalase system is also readily metabolized to pyruvate (Thornalley, 1996) which is utilized by a variety of processes such as glycolysis. Thus, the accurate quantification of D-lactate as a direct measure of MG metabolism may prove more difficult than initially anticipated. In the kidney of SD rats, we observed increased MG levels in the L- and D-Arg groups despite no change in glyoxalase I expression or activity. This may be due to excess MG being excreted by the kidneys into the urine as studies have shown radioactive MG to be excreted unchanged in the urine of rats, dogs and monkeys (Oliverio et al., 1963). Additionally, D-lactate was increased in the kidneys by D-Arg (500 mg, Fig. 3.40). This is most likely due to D-lactate being excreted into the renal tubule and reabsorbed by the kidneys (Thornalley, 1993).

When we supplemented ZDF rats with 1000 mg/kg/day of L-Arg we observed more successful results than in the SD rats. For example, in ZOC rats, MG levels were significantly increased in the plasma as compared to lean controls (Fig. 3.38). This was an expected effect as MG levels are

known to be increased in disease states such as T2DM (Dhar et al., 2011). When L-Arg (1000 mg) was given, we saw a significant decrease in plasma MG levels as compared to ZOC. In fact, plasma MG levels were similar to those observed in the ZLC group (Fig. 3.38). Furthermore, L-Arg (1000mg) maintained MG levels in all other organs except the lungs, to levels comparable to the lean controls (Fig. 3.39). Oppositely, D-Arg (1000 mg) tended to increase MG levels as compared to the ZLC. This was observed in the liver, lungs and kidney (Fig. 3.38). Increased MG levels by D-Arg (1000 mg) was an unexpected effect as this isomer was as effective of a scavenger as L-Arg *in vitro* (Dhar et al., 2012). Oddly, unlike in the SD rats, glyoxalase I expression was unchanged in the liver, kidney or ileum (Fig. 3.35). Finally, D-lactate levels remained unchanged in the plasma, urine or ileum (Fig. 3.41). D-Arg (1000 mg), however, did significantly increase D-lactate levels in the kidney (Fig. 3.41). This effect was also observed in SD rats as discussed above.

As previous work in our lab has linked increased MG levels with impaired glucose tolerance and insulin resistance (Dhar et al. 2011; L. Wu, 2008) we sought to understand if supplementing ZDF rats with MG scavengers, L- and D-Arg could ameliorate these hallmark characteristics of T2DM. A fasting blood glucose test is often performed to diagnose prediabetes or T2DM. We found that Zucker obese controls had significantly increased fasting blood glucose as compared Zucker lean controls (Fig. 3.42). This increase in fasting blood glucose was not ameliorated by L- or D-Arg (1000 mg). In agreement with these results, studies on the efficacy of L-Arg alone to improve fasting blood glucose have been largely unsuccessful (Jabłecka et al., 2012). However, L-Arg has proven to be successful in improving fasting blood glucose in diabetic patients when used as an adjunct to a healthier lifestyle (Lucotti et al., 2006). Lucotti *et al*. (2006) found that patients taking L-Arg in addition to consuming a healthy diet and committing to regular exercise had marked improvements over those on the same diet and exercise regime taking a placebo. We also performed an oral glucose tolerance test. We found that Zucker obese controls had impaired glucose tolerance as compared to lean controls and that this again was not improved *via* L-or D-Arg (1000 mg) supplements (Fig. 3.42).

Interestingly, fasting blood insulin was not significantly different in the Zucker obese controls compared to Zucker lean controls (Fig. 3.43). Again, no improvement was observed when L- or D-Arg (1000 mg) was given. Similarly, no significant differences in insulin levels were observed between any group in the oral glucose tolerance test (Fig. 3.43). Similar results have been found in human studies. For example, Piatti *et al.* (2001) did not observe improvement in serum insulin

levels but did observe cardiovascular improvements similar to those observed in our study (Fig. 3.13). Thus, we saw a significant decrease in MAP in L-Arg (1000 mg) supplemented ZDF rats (Fig. 3.13) and Piatti *et al.* noted improved forearm blood flow and decreased peripheral vascular resistance. Thus, it appears that arginine supplements are more effective in ameliorating cardiovascular symptoms of T2DM than improving glucose tolerance and insulin sensitivity. Finally, we assessed the glucose/insulin ratio in ZDF rats and found it was significantly increased by D-Arg (1000 mg) as compared to both obese and lean controls (Fig. 3.44). While the D-Arg group had similar fasting glucose levels as the ZOC and ZOA groups it did have lower fasting insulin levels. Usefulness of the glucose/insulin ratio is controversial (Quon, 2001), but this result would indicate a superior insulin sensitivity as compared to ZLC and ZOC (Fig. 3.44).

4.1 CONCLUSIONS

To the best of our knowledge, such a comprehensive study on the physiological effect of oral L- and D-Arg supplements has not been previously performed. From this study a few key conclusions could be made. First, oral arginine supplements do not alter arginine levels in the plasma or organs/tissues of SD or ZDF rats long term. They do, however, significantly downregulate the expression of the CAT-1 transporter which may affect the absorption of other biomolecules.

L-Arg significantly increased eNOS expression in both SD and ZDF rats. Despite increased eNOS expression in both models, only the pathological model – ZDF rats, showed improvements in MAP as a result of L-Arg supplementation. This supports the idea that arginine supplements are mostly effective in a disease state. Endothelial function also appeared to be improved in ZDF rats where L-Arg was given. Repeating this study with a larger sample size would most likely yield statistically significant results. The significant effect of D-Arg on eNOS and other enzymes demands a closer look at its possible enzyme interactions and mechanisms. Arginase often acts in opposition to eNOS and is found to have increased activity in disease states. While L-Arg decreased arginase expression in the ileum in SD rats, it significantly increased arginase expression in the ileum in ZDF rats, which supports the arginine paradox (Dioguardi, 2011). L-Arg also significantly increased ADC expression and as a result polyamine production in the liver, underscoring the need for caution when oral arginine supplements are solely taken for beneficial nitric oxide mediated cardiovascular effects.

In respect to MG scavenging, arginine supplements again proved more useful in a pathological model. Only in the ZDF rats did we observe a significant reduction in plasma MG levels *via* L-Arg. In most organs L-Arg was able to reduce MG levels. Oppositely, D-Arg tended to increase them, creating a case against this isomer as an *in vivo* scavenger. Finally, neither L- or D-Arg supplements were able to improve glucose tolerance or insulin responses in ZDF rats.

4.2 SIGNIFICANCE

The main strength of my study is that it was an all-encompassing investigation into how both L- and D-Arg supplements affected arginine metabolic pathways, basal MG levels, the glyoxalase system and endpoints such as MAP in both a physiological and pathological model. Our study supports their effectiveness in reducing blood pressure in hypertensive states. One of the main significant effects which was observed in SD as well Zucker rats with either 500 or 1000 mg dose was the significant decrease in ileum CAT-1 expression, as this transporter is required for the absorption of the essential amino acid lysine. Furthermore, downregulation of this transporter would most likely decrease the bioavailability of the arginine dose. Thus, increasing the dose would partly be negated by a decrease in CAT-1 and absorption, but a correlation of the magnitude of dose increase and the magnitude of decreased bioavailability needs to be established to give a proper dose recommendation for an oral dose.

Perhaps the most novel finding was in respect to D-Arg, a supposedly inert isomer, which as a consequence is rarely investigated. The possible molecular interactions of D-Arg with L-Arg metabolizing enzymes are warranted. Furthermore, deficiencies in arginine metabolizing enzymes are associated with a wide variety of conditions such as hyperargininenemia (Crombez & Cederbaum, 2005), endothelial dysfunction (Nakayama et al., 2009) , CNS and muscle dysfunction (Stockler-Ipsiroglu et al., 2015). Thus, when taking oral arginine supplements, one should err on the side of caution.

Arginine supplements are not strictly regulated with no established dose, and are heavily pushed by many distributors for their presumed beneficial vasodilating effect and improved aerobic exercise performance. This means that even healthy individuals and athletes are encouraged to take them. The results of our study highlight their effects on other enzymes and their metabolites which calls for more studies to establish safe doses and intake duration Dioguardi (2011) states that "*Arginine metabolism is very complex, and equally complex and highly interregulated is the*

availability of arginine for the various metabolic pathways in which this AA is involved. Exogenous arginine supplementation should be avoided, because it does not resolve the reasons underlying its excess consumption or blunted synthesis, and, on the contrary, may actually worsen them. The usual paradigm of clinical nutrition, assuming that what is lacking should be provided, may here prove to be wrong." This statement points out that not every deficiency can be solved by supplying more substrate and that furthermore supplementation may cause more harm than good. One cannot control where exogenous arginine will distribute, and which enzymes will preferentially metabolize it. Thus, in agreeance with the above statement, I believe pinpointing exactly which part of arginine metabolism is broken and remediating it would prove a more effective and safer solution than aimlessly supply exogenous arginine.

4.3 LIMITATIONS OF THE STUDY

The main advantage of our study was also the source of some limitations. My study was performed *in vivo* which allowed for a more holistic understanding of the physiological impact of L- and D-Arg supplements. However, interplay between the arginine metabolic pathways and other metabolic pathways at some points made forming definitive conclusions difficult. For example, the metabolic product of MG detoxification, D-lactate, raised some problematic results. One would assume that decreased MG levels would result in increased D-lactate levels or *vice versa*. However, this was not always the case. D-lactate is readily converted to pyruvate, can be excreted in the feces in addition to the urine and produced by intestinal bacteria. Assessing pyruvate levels, fecal D-lactate levels and if possible, rate of D-lactate production by bacteria may have helped produce a more complete picture of MG metabolism. Additionally, MG can be converted to L-lactate, thus quantifying plasma and organ/tissue levels of L-lactate would also be useful. This same issue arises when assessing metabolites common to multiple pathways such as urea, L-ornithine, L-citrulline and polyamines. Additionally, outside players such as ADMA can affect arginine metabolic pathways. ADMA is known to be a natural potent inhibitor of NOS enzymes. Levels of ADMA have been shown to be increased in individuals with disease states such as T2DM and hypertension. It would have been useful to measure ADMA levels in order to understand if increased levels of ADMA may have explained the lack of decreased MAP despite enhanced eNOS expression in some treatment groups.

Another issue with my study was not knowing the bioavailability of the dose being given and the immediate distribution. Studies on the pharmacokinetics of L- and D-Arg are far and few between. The current best guess of the bioavailability of oral L-Arg is ~40%. The bioavailability of D-Arg has not been investigated. It would have been extremely useful to have known exactly how much of the dose being given was reaching the bloodstream. Decreased bioavailability of L-arginine is most likely due to metabolism in the intestinal wall and the liver during first pass, but maybe measurement of fecal excretion levels of arginine may have been useful. Furthermore, measuring arginine levels in the feces may have help provide a better picture of how much arginine was reaching the bloodstream and how much remained in the external gastrointestinal system. Additionally, the distribution of arginine immediately after ingestion would have aided in interpreting results. Arginine levels were analyzed in the plasma and various tissues/organs. However, the plasma and tissues/organs were harvested and stored many hours after the rats ingested their supplemented water. These results showed no significant changes between treatment and control groups. This is in line with other studies in which alterations in arginine levels were not detected more than four hours after arginine administration. It appears that the immediate distribution of arginine is important to fully understand the effects of arginine supplements. For example, the ileum often showed the greatest changes in enzyme expression/activity and metabolite levels. This effect was presumably due to the ileum receiving the most arginine, a phenomenon which could have been confirmed by testing for acute distribution. Thus, performing pharmacokinetic tests to better understand where arginine is going in those first few hours may help explain why some organs showed greater changes in response to arginine than others. Finally, the ideal way to administer the oral dose as a bolus would be by gavage, which would more closely resemble humans taking supplements as a single dose three or four times a day. However, administration by oral gavage every day for 4 weeks or more would be very stressful for rats and add a confounding factor, and at the same time would increase the risk of mortality by inadvertent administration in the trachea and lungs.

4.4 FUTURE DIRECTIONS

Future work on this project would begin with investigating the pharmacodynamic effect of the 1000 mg/kg/day dose of oral L-and D-Arg in male SD rats. Essentially all the western blots, assays and HPLC analysis performed in Phase Ia would be completed in Phase Ib. This would allow us to

understand if a higher dose has a greater effect on arginine metabolic pathways. Additionally, it would allow us to perform a risk-benefit assessment. For example, does increasing the dose and duration of treatment increase beneficial effects such as lowering blood pressure *via* the eNOS pathway or does it increase harmful effects such as enhancing arginase activity? If both beneficial and harmful effects are enhanced at the 1000 mg/kg/day dose do the benefits outweigh the adverse effects? By investigating all four arginine metabolic pathways as well as the glyoxalase-MG pathways we can better assess the merit and safety of increasing the dose. Additionally, the two remaining pathways, ADC and AGAT, will need to be investigated in the ZDF rats to allow us to more fully understand the physiological effect of arginine supplements in a diabetic model. As MG derived AGEs are increased in diabetes it would also be interesting to investigate if arginine supplements are able to decrease AGEs in ZDF rats.

Finally, as discussed above one of the main limitations of my study was the lack of pharmacokinetic studies on oral L- and D-Arg. Thus, the next phase in this project would be to administer an acute dose of 500 mg/kg and 1000 mg/kg of L-and D-Arg *via* an i.v. bolus and oral gavage. This would allow us to calculate pharmacokinetic parameters including area under the curve for plasma drug concentration, systemic clearance, renal clearance, mean residence time, apparent volume of distribution, half-life, bioavailability, maximum plasma concentrations and time to C_{max} of each isomer. Having information on the pharmacokinetic properties of each isomer in addition to the pharmacodynamic effects would allow for more concrete conclusions to be drawn.

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