L-ARGININE TRANSPORT AND ENDOTHELIAL DYSFUNCTION IN CHRONIC KIDNEY DISEASE: THE ROLE OF EXERCISE

by

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A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Applied Physiology

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"If we could give every individual the right amount of nourishment and exercise, not too little and not too much, we would have found the safest way to health."

-Hippocrates, C. 450 B.C.

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DEDICATION

This dissertation is dedicated to my Dad, the original Dr. Martens, who has taught me the value of hard work and perseverance.

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LIST OF ABBREVIATIONS

AAU, arginase activity unit

ACE, angiotensin converting enzyme

ACH, acetylcholine

ADMA, assymmetric dimethylarginine

AI, 5/6 ablation/infarction

ANOVA, analysis of variance

BEC, S-(2-Boronoethyl)-L-cysteine hydrochloride

BH_4, tetrahydrobiopterin

BUN, blood urea nitrogen

CaM, calmodulin

CAT-1, cationic amino acid transporter-1

CKD, chronic kidney disease

CVD, cardiovascular disease

DBP, diastolic blood pressure

DDAH, dimethylarginine dimethylaminohydrolase

EDR, endothelium-dependent relaxation

eGFR, estimated glomerular filtration rate

eNOS, endothelial nitric oxide synthase

ESRD, end-stage renal disease

FMD, flow-mediated dilation

GFR, glomerular filtration rate

GSI, glomerulosclerosis index

L-NAME, L-N^G-nitro-L-arginine methyl ester

L-NMMA, L-N^G-monomethyl Arginine

MAP, mean arterial pressure

NAD(P)H, nicotinamide adenine dinucleotide phophate

NO, nitric oxide

NOS, nitric oxide synthase

NOx, nitrates & nitrites

O_2^-, superoxide

$ONOO^-$, peroxynitrite

PAN, puromycin aminonucleoside

PE, phenylephrine

PKCα, protein kinase C alpha

PRMT, protein methyl transferase

SBP, systolic blood pressure

sGC, soluble guanylyl cyclase

SLC7, solute carrier family 7

SNP, sodium nitroprusside

SOD, superoxide dismutase

ABSTRACT

Chronic kidney disease (CKD) affects more than 26 million people in the United States and is associated with an elevated risk of cardiovascular disease (CVD) that persists even in the absence of traditional cardiovascular risk factors. Impairments to the vascular endothelium precede the development of atherosclerosis and have been extensively studied as a potential therapeutic target to treat CVD, however few studies have been performed in CKD. A reduction in the bioavailability of the vasodilator nitric oxide (NO) contributes to CVD-related mortality in CKD and is likely related to a reduced availability of the NO substrate L-arginine. Interestingly, the use of L-arginine in studies of endothelial dysfunction in late-stage CKD has produced mixed results unlike other diseases where it has been largely effective.

Evidence from cell culture studies suggests that urea and other uremic toxins inhibit L-arginine uptake into endothelial cells and may provide a unique explanation for why L-arginine treatment has been ineffective in late-stage CKD. Additionally, competition for L-arginine from the enzyme arginase as well as increased oxidative stress are possible mechanisms by which NO production and bioavailability are reduced. Physical activity has been shown improve vascular function in patients with CVD through reductions in oxidative stress and improvements in NO synthesis, and may be an effective therapy to reverse vascular dysfunction in CKD. Increased physical activity has also been shown to augment L-arginine uptake in the forearm vasculature of heart failure patients. The known cardiovascular benefits of aerobic exercise combined with its potential to improve L-arginine uptake make increased

physical activity an attractive therapy to reduce cardiovascular risk in CKD. Our overall hypothesis was that a deficiency in L-arginine availability contributes to endothelial dysfunction in rats with chronic kidney disease and that this impairment would be restored by increased physical activity.

In our first series of experiments, we explored the mechanisms of impaired endothelium-dependent relaxation (EDR) in a rat model of moderate to severe CKD. The enzyme arginase has been implicated as a potential contributor to vascular dysfunction by contributing to the consumption of L-arginine, reducing its availability for NO production. The purpose of this study was to determine if arginase contributes to endothelial dysfunction in the 5/6 ablation/infarction (AI) rat model of CKD. 12 week old male Sprague Dawley rats underwent AI surgery to induce CKD, or a SHAM surgery to serve as a control. EDR of aortic rings to acetylcholine was significantly impaired in AI animals vs. SHAM after 8 weeks (Emax; 56.17 ± 7.82 vs 100.85 ± 5.55, $p<0.05$; LogEC50; -5.67 ± 0.14 vs -7.74 ± 0.25, $p<0.05$), and was not improved by arginase inhibition (BEC) alone or in combination with L-arginine. Additionally, scavenging of superoxide (Tempol, Tempol + L-arginine, Tempol + L-arginine + BEC) was not effective suggesting that a mechanism independent of oxidative stress contributes to EDR in moderate to severe CKD. Neither arginase enzyme activity (0.012 ± 0.004 vs. 0.014 ± 0.006) or protein expression of either isoform of arginase (arg1: 0.79 ± 0.03 vs. 1.00 ± 0.11; arg2: 0.85 ± 0.18 vs. 1.00 ± 0.25) was increased in AI animals vs. SHAM, confirming that arginase does not contribute to impaired EDR in CKD. Aortic uptake of radio-labeled L-arginine was attenuated in AI animals vs. SHAM (47.06 ± 12.2 vs. 100 ± 16.3, $p <0.05$) and was associated with a reduced protein expression of the L-arginine transporter CAT-1

(0.37 ± 0.06 vs. 1.0 ± 0.17, $p<0.05$). These data suggest that arginase does not contribute to endothelial dysfunction in CKD; however, impaired L-arginine transport may play an important role in diminishing substrate availability for NO production leading to endothelial dysfunction.

Our second set of experiments was designed to reverse the impairment in endothelial function using a voluntary wheel running intervention. We tested the effect of 4 weeks of voluntary wheel running (RUN) and/or ARG supplementation on vascular function in rats with CKD. We hypothesized that ARG intervention alone would be ineffective due to impaired L-arginine transport and that RUN would improve EDR, in part due to improved utilization of L-arginine. Based on this hypothesis, we also predicted that the greatest improvement in EDR would be observed when animals were treated with ARG and RUN in combination. 12 week old male Sprague Dawley rats underwent either AI surgery to induce CKD, or a SHAM surgery as a control. CKD animals either remained sedentary (SED) or received one of the following interventions: supplemental ARG (1.25 g/L in drinking water), RUN, or combined RUN+ARG. Interventions began 4 weeks after surgery to allow time for disease progression. Animals were sacrificed 8 weeks after surgery and EDR was assessed by dose response to acetylcholine in aortic rings. EDR was significantly impaired in SED vs. SHAM animals after 8 weeks, demonstrated by an attenuated area under the curve (AUC; 44.56 ± 9.01 vs. 100 ± 4.58, $p<0.05$) and reduced maximal response (Emax; $59.9\% \pm 9.67$ vs. $94.31\% \pm 1.27$, $p<0.05$). AUC was not improved by ARG treatment but was significantly improved above SED animals in both RUN and RUN+ARG treated animals. Emax was elevated above SED in RUN+ARG animals only. CAT-1 protein expression was impaired in SED

animals and was not improved by any of the interventions. Aortic uptake of radio-labeled L-arginine was attenuated in SED and ARG-treated animals and was significantly improved in both RUN and RUN+ARG animals. PKCα has been implicated as a contributor to reduced L-arginine transport and was shown to be significantly elevated in SED animals, but returned to similar levels as SHAM animals in both RUN and RUN+ARG treated animals. The results indicate that voluntary wheel running is an effective therapy to improve vascular function in CKD, in part through improvements in L-arginine uptake and suggest that exercise may be a beneficial adjunct therapy to address cardiovascular disease in patients with CKD.

Chapter 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Cardiovascular Disease in Chronic Kidney Disease

Chronic kidney disease (CKD) is a major public health concern that results in progressive and irreversible loss of renal function. CKD affects over 26 million people in the United States [35] and contributes to rising healthcare costs [137]. Guidelines from the Kidney Disease Outcomes Quality Initiative (K/DOQI) place patients with CKD into one of five stages (Table 1.1) based on glomerular filtration rate (GFR) [34, 103]; however, symptoms typically develop slowly such that most patients are unaware that they have the disease until after they have advanced to higher stages [111]. There is a well-known association between CKD and cardiovascular disease (CVD) that contributes to more than half of all deaths in patients with CKD [146]. This association is so great that patients are more likely to die of CVD than to ever progress to end-stage renal disease (ESRD) [73, 126]. The risk of developing cardiovascular disease in CKD persists even in patients who lack traditional CVD risk factors such as old age, high HDL cholesterol, and diabetes mellitus [126]; therefore, it is important to understand the mechanisms of cardiovascular disease in CKD.

Patients with CKD exhibit hypertension, cardiomyopathy, atherosclerosis, arterial stiffening, and endothelial dysfunction [126]. Impairments to the vascular endothelium precede the development of atherosclerosis [38, 136] and have been

extensively studied as a potential therapeutic target to treat CVD; however, few studies have explored the specific mechanisms of endothelial dysfunction in CKD. The majority of previous research has focused on vascular dysfunction in patients with ESRD and in patients receiving dialysis. Although these studies have been important, the majority of patients with CKD die of CVD before needing dialysis [53]; therefore, there is an urgent need to research the mechanisms of vascular dysfunction earlier in the progression of CKD.

Table 1.1 Stages of CKD

Stage	Description	GFR, mL · min^{-1} per 1.73 m^2
1	Kidney damage with normal or increased GFR	≥ 90
2	Kidney damage with mildly decreased GFR	60-89
3	Moderately decreased GFR	30-59
4	Severely decreased GFR	15-29
5	Kidney failure	< 15 or dialysis

Adapted from National Kidney Foundation K/DOQI clinical practice guidelines for chronic kidney disease [103]

1.2 The Vascular Endothelium

The interior lining of blood vessels consists of a single layer of cells known collectively as the vascular endothelium. In addition to acting as a barrier between the intravascular space and the interstitium, the endothelium plays a critical role in regulating vascular homeostasis by contributing to the control of leukocyte adhesion

and infiltration [84], smooth muscle proliferation [130], and vascular tone [55, 97]; all of which have important implications for maintaining cardiovascular health [141]. Among the many important functions of the endothelium is the production and release of the vasodilator nitric oxide (NO) [71]. As depicted in Figure 1.1, endothelium-derived NO is synthesized from the amino acid L-arginine by the enzyme, endothelial nitric oxide synthase (eNOS) [97]. Once produced, NO diffuses through the basal membrane of the endothelium into the vascular smooth muscle where it activates the enzyme, soluble guanylyl cyclase (sCG), leading to the conversion of GTP to cyclic GMP [97]. The production of cyclic GMP leads to a signaling cascade that ultimately results in smooth muscle relaxation through inactivation of myosin light chain kinase (MLCK) thus preventing cross bridge formation and force generation within the vascular smooth muscle [22].

1.2.1 Endothelial Nitric Oxide Synthase (eNOS)

There are three known isoforms of the nitric oxide synthase (NOS) family of enzymes: Neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) [2]. Of these, the latter has been identified to be the most abundant in the vascular endothelium and is therefore the predominant contributor to the synthesis of endothelial-derived NO [2]. Regulation of eNOS activity can occur through transcriptional, post-transcriptional, and post-translational modifications that all result in alterations in overall NO production [88]. In addition, substrate and cofactor availability, endogenous NOS inhibitors, and regulation of NO bioavailability contribute to the ability of eNOS to regulate vascular homeostasis. The functional form of eNOS exists as a dimer and requires the support of several co-factors and

stabilizing proteins to maintain its activated structure. These include redox-sensitive cofactors such as flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), as well as tetrahydrobiopterin (BH_4), heme, and calmodulin (CaM). Adequate availability of these cofactors is important for maintenance of NO production through increased stabilization of the eNOS dimer [2] while a deficiency of one or more of these co-factors can result in eNOS uncoupling and reduced NO synthesis.

The subcellular localization of eNOS is primarily confined within small invaginations in the endothelial membrane known as caveolae. Interaction of eNOS with the scaffolding protein caveolin-1 anchors the enzyme near the cellular surface where it is most available to interact with additional cellular machinery required for NO synthesis [76]. Intracellular calcium that arises from activation of mechanosensitive pathways or agonist binding to endothelial cell surface receptors, binds to calmodulin (CaM) and dislocates caveolin-1 from its binding site on eNOS [95]. Once detached from caveolin-1, eNOS is bound by the chaperone protein heat shock protein 90 (Hsp90), providing additional stabilization and promoting NO production [116]. Hsp-90 recruits and maintains binding of the kinase Akt, which phosphorylates eNOS at serine-1177 resulting in sustained NO production [21]. The regulation of eNOS expression and activity is highly influenced by external mechanical stimuli acting against the endothelium resulting in both short-term and long-term (adaptive) alterations in NO synthesis.

1.2.2 L-arginine Transport

In addition to modifications in eNOS expression and activity, the synthesis of NO is dependent upon an adequate supply of the eNOS substrate, L-arginine.

Delivery of extracellular L-arginine to the endothelium occurs primarily through the cationic amino acid transporter, CAT-1. Classified as a member of the solute carrier family 7 (SLC7), CAT-1 contains 14 transmembrane domains and is responsible for the transport of all cationic amino acids in a pH and sodium-independent manner [32, 44]. Similar Km values between 100-150 μM have been reported of CAT-1 for the amino acids L-arginine, L-lysine, and L-ornithine with a strong preference for transport into the cells, although CAT-1 can also contribute to efflux of amino acids [32]. In addition to the transport of amino acids, CAT-1 is also capable of transporting methylated analogs of L-arginine including the endogenous NOS inhibitor, ADMA [139].

CAT-1 is ubiquitously expressed in all tissues of the body with the exception of the adult liver [23]. In vascular tissue, it is primarily localized in close proximity to eNOS within endothelial cell caveolae. CAT-1 has been shown to co-localize with eNOS [87, 94] and may be responsible for facilitating the delivery of L-arginine for NO production. Acute alterations in L-arginine transport are regulated by similar mechanisms as NO production such as agonist binding [106] and endothelial shear stress [115] suggesting a coupling between substrate delivery and NO synthesis. The subcellular localization of CAT-1 is regulated by protein kinase C alpha (PKCα) whereby increased PKCα expression at the endothelial membrane results in reduced L-arginine transport activity [83]. In addition to alterations in transport activity, regulation of L-arginine transport may occur through alterations in CAT-1 protein expression, substrate affinity, and subcellular localization.

1.3 Endothelial Dysfunction in CKD

A reduction in the bioavailability of nitric oxide has been shown to contribute to cardiovascular disease mortality in patients with CKD [89]. Mechanisms that disrupt the synthesis or release of NO contribute to endothelial dysfunction and ultimately lead to the development of hypertension and atherosclerosis [136] and contribute to reductions in eGFR [109]. Endothelial dysfunction has been shown to occur in both humans and animals with CKD [4, 11, 36, 63, 164] and may explain the increased cardiovascular disease risk associated with CKD; however, the specific mechanisms have not yet been fully explained. Increased production of reactive oxygen species (ROS) leading to oxidative stress, and a disruption in the synthesis, delivery, and utilization of L-arginine for NO production appear to play a definitive role in the pathogenesis of endothelial dysfunction in CKD and their mechanisms are reviewed below.

1.3.1 Oxidative Stress

The pathological disruption of redox signaling, known collectively as oxidative stress, is a widely studied contributor to endothelial dysfunction across a variety of cardiovascular pathologies [64]. Nitric oxide itself is a free radical and is therefore a susceptible target of redox reactions that result in reduced NO bioavailability [59] (Figure 1.1). Oxidative stress is present in patients with end-stage renal disease and in patients receiving hemodialysis [165] and appears to contribute to the development of endothelial dysfunction that worsens with progressive loss of renal function [3, 4, 165].

The mechanisms by which oxidative stress contributes to endothelial dysfunction occur primarily through NOS dependent pathways. Cross et al. (2003) observed a reduction in acetylcholine-induced forearm blood flow (venous occlusion plethesmography) in pre-dialysis patients with ESRD (GFR < 20 mLmin) [37]. This impairment was ameliorated following the infusion of the antioxidant ascorbic acid through the brachial artery, suggesting that oxidative stress contributed to endothelial dysfunction in these patients. Importantly, forearm blood flow was not restored when ascorbic acid was co-infused with the NOS inhibitor L-NMMA, or the endothelium-independent dilators glyceryltrinitrate (GTN) or sodium nitroprusside (SNP). This indicates that the impaired forearm blood flow in these patients was due to a reduction in endothelium-derived NO and not an impairment in vascular smooth muscle function.

Oxidative stress also contributes to endothelial dysfunction in earlier stages of CKD [5, 102]. The forearm blood flow response to an arterial infusion of methacholine was attenuated in patients with moderate renal impairment, and was accompanied by an increase in lipid hydroperoxides (LOOH) and an increased ratio of oxidized to reduced glutathione (GSSG/GSH) [5]. These markers of oxidative stress were correlated to both serum creatinine and forearm blood flow suggesting that oxidative stress contributes to endothelial dysfunction even in moderate stages of CKD.

ROS decrease NO production and bioavailability through a variety of mechanisms. The combination of NO with the highly reactive oxidant, superoxide (O_2^-) results in the formation of the radical peroxynitrite ($ONOO^-$) and reduces the amount of NO available for vasodilation (Figure 1.1) [59]. In addition to reducing NO

bioavailability, peroxynitrite formation contributes to nitrositive cellular damage. Vaziri et al., measured increased nitrotyrosine abundance, a marker of nitrositive protein modification, in aorta, heart, liver, and plasma of 5/6 nephrectomized rats [154]. Nitrotyrosine levels were restored to normal levels in rats treated with the antioxidant vitamin E [154]. In another study, scavenging of superoxide with the SOD mimetic Tempol prevented the increase in systolic blood pressure in rats within the first 10 days following 5/6 nephrectomy [63]. Likewise, vascular responsiveness to acetylcholine was impaired in these rats and was improved when treated with exogenous SOD, suggesting that superoxide mediates the progression of vascular dysfunction and hypertension in CKD.

The specific source of superoxide production in CKD has not been completely identified; however, activation of NAD(P)H oxidases by angiotensin II has been suggested as a likely contributor to oxidative stress in CKD [77]. Few studies have examined the role of inhibition of the renin-angiotensin system on endothelial-dependent dilation in CKD. Vavrinec et al. demonstrated that ACE inhibition with Losartan reduced proteinuria and blunted systolic blood pressure in rats that underwent 5/6 ablation/infarction [153]. In humans with stage 1 diabetic CKD, endothelium-dependent dilation (assessed by brachial artery FMD) was improved following short term ACE-inhibition [166]; therefore, it appears that elevated levels of angiotensin II in CKD may contribute to increased superoxide production and endothelial dysfunction in CKD. Additional free radical production may occur from increased activity of xanthine oxidase, however the role of this enzyme in humans with CKD is not well understood. Treatment with the xanthine oxidase inhibitor allopurinol slowed the progression of CKD, and reduced cardiovascular risk in

patients with CKD (eGFR < 60 ml/min) [56]; however, it is unclear if these beneficial effects were due to an actual decrease oxidative stress or a reduction in serum uric acid concentration.

In addition to reduced bioavailability, oxidation of the cofactor BH_4 contributes to the destabilization and uncoupling of the eNOS dimer (Figure 1.1). Uncoupled eNOS produces superoxide at a higher rate than NO, contributing to the increase in oxidative stress and the further decline in NO bioavailability [57, 152]. Supplementation with BH_4 has been shown to lower systolic blood pressure and reduce proteinuria in 5/6 nephrectomized rats [112]. In another study, endothelium-dependent relaxation of aortic rings from 5/6 nephrectomized rats was restored in isolated vessels treated with BH_4 [164]. These vessels were also associated with an increase in superoxide production, which was ameliorated by treatment with L-NAME suggesting that the source of oxidative stress was uncoupled eNOS. Treatments aimed at restoring eNOS coupling may therefore be effective at reducing endothelial dysfunction in CKD.

Free radical production is typically buffered by the endogenous antioxidant defense system. Plasma and erythrocyte levels of the endogenous antioxidant enzymes glutathione peroxidase (GSH-px), superoxide dismutase (SOD), and catalase have been shown to be significantly lower in pre-dialysis patients with ESRD compared to healthy control subjects [25]. In addition, antioxidant levels were slightly higher in dialysis patients than in pre-dialysis patients suggesting that removal of ROS from the blood may improve antioxidant defense [25]. Endogenous antioxidants have also been shown to be attenuated in earlier stages of CKD with levels of erythrocytic GSH-px, SOD, Zn, and Cu impaired as early as stage 1 CKD (GFR < 90 ml/min)

[165]. These studies suggest that an impaired antioxidant defense system may contribute to elevated levels of free radicals in CKD.

Oral supplementation with antioxidants has been largely ineffective at reducing cardiovascular events in other diseases [151]; however, antioxidant use has been shown to reduce cardiovascular events in CKD and hemodialysis[144]. Hemodialysis patients that received 800 IU/day of vitamin E had fewer primary cardiovascular end-points than placebo-treated controls in the Secondary Prevention with Antioxidants of Cardiovascular Disease in Endstage Renal Disease (SPACE) trial [14]. Antioxidant therapy has also been shown to improve endothelial function in patients with earlier stages of CKD. In the Antioxidant Therapy in Chronic Renal Insufficiency (ATIC) study, brachial artery FMD was improved in patients with mild to moderate renal impairment following an antioxidant intervention [102]. The intervention consisted of three stages of drug treatment in which patients received pravastatin, vitamin E, and homocysteine-lowering therapy, sequentially in 6-month intervals. The observed improvement in vascular function occurred despite the continued progressive decline in renal function. These studies suggest that the use of antioxidants may be an effective therapy to reduce oxidative stress and improve cardiovascular function in CKD.

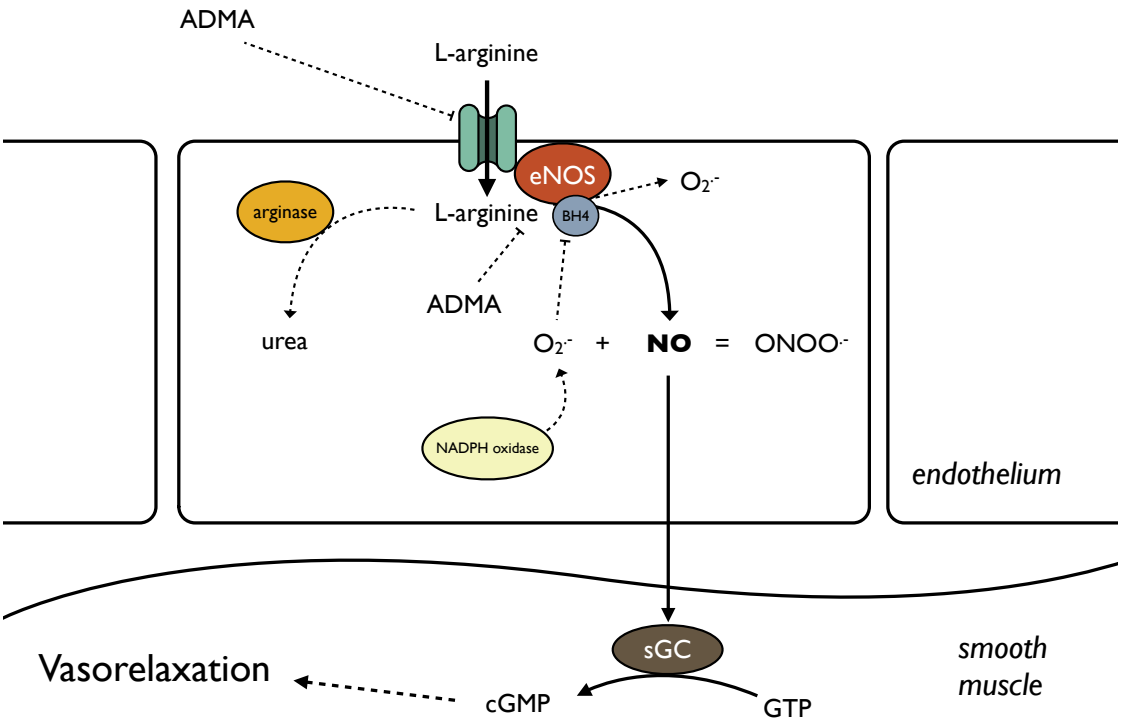

Figure 1.1: **Potential Mechanisms of Endothelial Dysfunction in CKD.** Nitric oxide (NO) is synthesized by the endothelial nitric oxide synthase (eNOS) from the amino acid L-arginine. NO diffuses into the vascular smooth muscle where it activates the conversion of GTP to cyclic GMP (cGMP) by the enzyme soluble guanylyl cyclase (sGC), initiating vasorelaxation [97]. Reduced availability of L-arginine due to impaired transport, competition from arginase, or competitive inhibition of eNOS by asymmetric dimethylarginine (ADMA) may contribute to endothelial dysfunction [9, 119]. Additionally, oxidative stress resulting from increased superoxide production can reduce NO bioavailability through the combination of NO with $O_2^{\cdot-}$ forming peroxynitrite ($ONOO^-$). Oxidation of the cofactor BH_4 can also lead to uncoupling of eNOS resulting in further superoxide production [57].

1.3.2 Impaired L-arginine Transport

In addition to oxidative stress, impairments to substrate delivery and availability for NO production contribute to endothelial dysfunction in CKD. Although the intracellular concentration of L-arginine exceeds its Michaelis-Menten (Km) constant for eNOS, treatment with exogenous L-arginine has been shown to paradoxically increase NO production [61] in a variety of clinical pathologies [15, 31, 33, 51, 110, 147] and in primary aging [15, 24, 66]. This phenomenon suggests that NO production relies on L-arginine from extracellular sources and implies a relative deficit in L-arginine availability under diseased conditions that is reversed by exogenous treatment. Such a deficit seems particularly likely as L-arginine metabolism has been shown to be impaired in CKD [27] with attenuation of L-arginine synthesis and transport as well as a relative reduction in intracellular L-arginine concentrations due to an increased production of the endogenous NOS inhibitor, assymetric dimethylarginine (ADMA) [26].

L-arginine is primarily synthesized from the precursor, L-citrulline, in proximal tubule cells of the kidney by a two step process involving the enzymes argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) [27]. The abundance of both of these enzymes was attenuated in kidney homogenates from rats that underwent renal mass reduction [27] and in rats with puromycin aminonucleoside (PAN)-induced CKD [26]. These animals also exhibited an elevation in plasma citrulline concentration suggesting that less citrulline was being converted to L-arginine.

In addition to impaired substrate synthesis, glomerular and endothelial L-arginine transport is impaired in CKD, resulting in a net decrease in the supply of L-

arginine for NO production [72, 90, 128, 158, 163]. The use of exogenous L-arginine to treat impaired renal production of L-arginine seems like an attractive therapy in patients with CKD; however, L-arginine treatment in CKD has been met with mixed results [11, 36, 164]. Chronic treatment with L-arginine for 9 weeks prevented the development of endothelial dysfunction in 5/6 nephrectomized rats [164]; however, acute infusion of L-arginine was not effective in pre-dialysis patients with severe renal failure [36]. These findings suggest that while L-arginine may prevent the development of vascular dysfunction early in the progression of CKD, it is less effective once CKD has already been established. These findings are supported by recent evidence suggesting a link between high levels of uremic toxins and impaired L-arginine transport [158, 163] as indicated by a maintenance of a normal plasma L-arginine concentration despite decreased synthesis [10, 119].

Transport of L-arginine into the endothelium occurs primarily through the cationic amino acid transporter, CAT-1, and has been shown to be inhibited in endothelial cells cultured in uremic plasma and in cells cultured in a synthetic solution containing uremic levels of urea [163]. The influx of extracellular urea through endothelial urea transporters (UT) appears to contribute to the attenuation of L-arginine transport as UT inhibition prevents the decline in L-arginine transport in cells treated with high levels of urea [158]. Impaired L-arginine transport has been confirmed in rat models of CKD in which aortic uptake of L[^{3}H]-arginine was shown to be impaired and related to a decrease in CAT-1 expression and activity [72, 90, 128]. Transport of extracelluar L-arginine into the endothelium is important for NO production [169] and CAT-1 has been shown to form a caveolar complex with the

NO-producing enzyme, eNOS [87, 94]. It is therefore plausible that NO synthesis is regulated in response to changes in CAT-1 expression and transport activity.

The regulation of CAT-1 has been linked to protein kinase Cα (PKCα) where an activation of PKCα results in reduced L-arginine transport activity [83]. While CAT-1 has been shown to contain 3 potential sites for phosphorylation by PKCα, Rotmann et al. (2004) demonstrated that direct phosphorylation is not necessary to mediate a reduction in transport activity. Mutation of the putative phosphorylation sites on CAT-1 did not alter the ability of PKCα to reduce L-arginine transport suggesting an indirect mechanism of inactivation [121]. Interestingly, part of the decline in transport activity appears to be the result of internalization of CAT-1 to the cytosol as stimulation of *X. laevis* oocytes with the PKCα activator, PMA, resulted in translocation of CAT-1 to the cytosol [121]. This inactivation may also play a role in the ultimate down regulation of CAT-1 protein expression as PKCα-dependent reductions in CAT-1 activity have been shown to be associated with ubiquitination, and subsequent targeting of the transporter for lysosomal degradation [156].

Evidence for a PKCα mediated decline in CAT-1 transport activity has been shown to occur in rats with CKD and was normalized by treatment with rosiglitazone through a mechanism that was independent of changes in CAT-1 protein expression [72]. In an earlier study by the same group, uremic rats treated with either atorvastatin or L-arginine following 5/6 nephrectomy demonstrated improved L-arginine transport that *was* associated with improved CAT-1 protein expression [128]. Taken together, these findings suggest that L-arginine transport can be altered via changes in both transporter activity and protein abundance. Treatments aimed at restoring CAT-1

expression and activity may therefore have a positive effect on vascular function by improving substrate delivery and NO production.

1.3.3 Competition from Arginase

In addition to impairments in cellular transport, L-arginine availability for NO production may be diminished by competition from the enzyme arginase. Such a mechanism has been demonstrated in primary aging [13, 80, 162] and hypertension [7] whereby inhibition of arginase resulted in improved endothelial function.
L-arginine is used by arginase as a substrate in the urea cycle and is thought to be diverted from eNOS when arginase activity is increased, resulting in impaired NO production (Figure 1.1). Systemic inhibition of arginase with a manganese (Mn^{2+})-free diet suppressed arginase activity in the liver and kidney of rats that underwent 5/6 ablation/infarction surgery and slowed the progression of renal impairment [124]. Though this treatment was effective, it is uncertain whether it would have slowed the progression of more advanced CKD where circulating levels of urea and other toxins are markedly increased.

Urea is normally excreted in the urine as a waste product of protein metabolism but is retained in the blood during kidney disease as a result of kidney damage. The increase in circulating urea associated with CKD may decrease the activity of arginase in a negative feedback manner such that any increase in arginase activity is suppressed with advancing CKD. This type of uremic inhibition has been demonstrated in liver homogenates of 5/6 nephrectomized rats [100]. More recently, Chen et al. (2012) demonstrated no increase in arginase expression in rats treated with a high dose of puromycin aminonucleoside (PAN) to induce severe CKD [26].

Interestingly, rats treated with a low dose of PAN as a model of moderate CKD demonstrated a significant increase in arginase expression. Data from our own lab confirm these findings by demonstrating no increase in the expression or activity of arginase in aortic tissue of animals with late-stage CKD [90]. Taken together, these studies suggest that arginase does not play a role in late-stage CKD, however its role earlier in the progression of CKD is worthy of future investigation.

1.3.4 Endogenous NOS Inhibition by ADMA

In addition to impaired substrate delivery, formation of the endogenous NOS inhibitor asymmetric dimethylarginine (ADMA) is increased in CKD [9, 79] and has been identified as an independent risk factor for CVD [134]. ADMA is produced as the result of post-transcriptional methylation of L-arginine residues by protein arginine methyltransferases (PRMTs) and is released in its free form following protein hydrolysis. The chemical structure of ADMA is close enough to that of L-arginine that it prevents NO production through competitive inhibition of eNOS (Figure 1.1). ADMA production is elevated in patients with chronic renal failure while its clearance by the kidney is impaired, resulting in a net increase in the plasma concentration of ADMA in patients with CKD [78, 149] and contributing to increased mortality and adverse cardiovascular events [118, 168].

Methylated arginines have been linked to impaired endothelial function. Brachial artery FMD was impaired in healthy individuals treated with the methylated arginine L-NMMA suggesting that a reduction in the ratio of L-arginine to methylated arginine competitively inhibits NO production *in vivo* [36]. Subsequent addition of L-arginine in these patients improved this balance and restored endothelial function.

While L-arginine treatment is an effective strategy to restore substrate balance relative to ADMA in other disease models, its effectiveness in CKD is questionable due to impaired L-arginine transport. It is therefore necessary to explore alternative strategies for reducing circulating levels of ADMA in kidney disease.

Interventions designed to lower ADMA production or increase ADMA clearance may be effective at improving endothelial function in CKD. Clearance of ADMA in the urine is impaired with renal damage and contributes to elevated plasma concentrations [78]; however, the primary reason for elevated ADMA in CKD is not explained by impaired urinary clearance alone [92]. Increased expression and activity of PRMT's, and reduced degradation of ADMA by dimethylarginine dimethylaminohydrolase (DDAH) likely account for the majority of accumulated ADMA in CKD [10]. PRMT expression and activity is increased in the presence of oxidized LDL cholesterol and results in increased production of ADMA in endothelial cells [17]. Intravenous infusion of the antioxidant N-acetylcysteine has been shown to reduce ADMA levels in patients receiving hemodialysis [145] while vitamin E therapy has been shown to reduce ADMA levels in predialysis patients with CKD [125] and may be an effective treatment strategy to restore endothelial function. PRMT expression was found to be increased in an animal model of CKD [92] and may be a potential therapeutic target to restore endothelial function in humans. In addition, ACE inhibition may reduce ADMA levels and contribute to improved endothelial function. One study showed that 3 months of treatment with the ACE inhibitor Ramipril (5 mg/day) or the angiotensin II receptor antagonist Valsartan (160 mg/day) reduced ADMA levels and restored FMD in patients with non-diabetic CKD [167].

Despite increased plasma concentrations in CKD, ADMA is most likely not responsible for impaired L-arginine transport [139, 143, 163]. Xiao et al. (2001) demonstrated that CAT-1 transport of L-arginine in cultured endothelial cells is not inhibited by uremic levels of ADMA (10μM) and is only impaired when ADMA is given at supraphysiological concentrations (0.1mM-2mM). This suggests that while ADMA may still inhibit NO production by competing with L-arginine for eNOS, it does not play a role in reducing L-arginine transport through competitive inhibition of CAT-1. ADMA is transported into endothelial cells through the CAT transport system [143, 150], however its entry into the endothelium in CKD is likely inhibited to a similar extent as L-arginine. The elevated plasma levels of ADMA have therefore been suggested as a marker of CKD, but not necessarily a contributor to endothelial dysfunction [143]. A more likely role of ADMA in the pathogenesis of endothelial dysfunction in CKD may be through increased intracellular production by PRMTs or reduced intracellular degradation by DDAH within endothelial cells. Whether increased intracellular ADMA is capable of inhibiting extracellular L-arginine entry is presently unknown. Overall reductions in CAT-1 activity in CKD may contribute to elevated intracellular concentrations of ADMA and ADMA/L-arginine ratio resulting in reduced NO production.

1.4 Exercise and Vascular Function in CKD

One potential method to improve endothelial function in CKD is through increased physical activity. Exercise is a well-known stimulus for improved cardiovascular function [58] and has been shown to restore vascular function in patients with cardiovascular disease [159]. Indeed, 2-4 weeks of exercise training in

rats has been shown to improve endothelial-dependent dilation through enhanced NO production [140] and increased eNOS expression [42, 131]. Exercise has also been shown to improve the endogenous antioxidant defense system [123] and increase stabilization of eNOS through upregulation of hsp90 [62]. It is now well understood that the primary stimulus for increased NO production is endothelial shear stress [58]; therefore, increased blood flow associated with exercise training likely improves vascular function through improved NO synthesis.

1.4.1 Shear Stress and NO Production

Tangential and circumferential shear stress resulting from the pulsatile nature of blood flow activate pathways that increase eNOS expression and activity in order to tightly regulate blood flow and maintain vascular homeostasis [8]. A biphasic increase in NO production occurs in endothelial cells in response to shear stress against the endothelial surface [85]. Upon the onset of shear forces, an immediate burst of NO is released followed by sustained synthesis lasting as long as shear forces are present [8]. This sustained NO synthesis is largely attributed to phosphorylation of eNOS at serine-1177 and serine-633 by the kinases Akt, PKA, PKC, and AMPK in a "calcium-independent" manner [8, 46]. Mechanisms of shear-induced production of nitric oxide are depicted in Figure 1.2.

Transduction of shear forces into endothelial-derived nitric oxide occurs through extracellular matrix proteins, integrins and adhesion molecules, mechanically sensitive ion channels and the cellular cytoskeleton [8]. For example, co-localization of eNOS with the platelet endothelial cell adhesion molecule (PECAM-1) has been shown to be important for Akt phosphorylaiton of eNOS in response to shear[52].

Shear related increases in NO production are largely considered to occur by a Ca^{2+}-independent mechanism as shear-induced NO production exceeds that obtained by maximal intracellular Ca^{2+} concentrations [8]. Nevertheless, at least some of the NO produced in response to increased shear stress can be attributed to changes in intracellular Ca^{2+} concentrations through the mechanical activation of membrane K^{+}, Cl^{-}, and Ca^{2+} ion channels [8].

In addition to acute increases in NO synthesis in response to shear stress, long-term improvements in endothelial NO production can occur in response to sustained increases in blood flow. The promoter region of the eNOS gene contains a "shear-stress response element" upon which transcription factors bind and promote the transcription of eNOS mRNA [39]. Activation of the transcription factors NFκB and and KLF2 through shear-dependent mechanisms have been linked to increases in NO production [39, 41]. Under normal conditions, transcription of eNOS is regulated by a negative feedback mechanism in which an overly abundant supply of NO deactivates NFκB via S-nitrosylation resulting in an inhibition of eNOS gene transcription [8]. Post-transcriptional regulation of eNOS occurs via stabilization of eNOS mRNA and has also been shown to be linked to mechanical stimulation. Cultured endothelial cells exposed to shear stress demonstrated a 3-fold increase in the half-life of eNOS mRNA that was associated with increased 3' polyadenylation [160]. Long-term adaptations to increases in shear stress occur with exercise training and have been shown to positively influence vascular function.

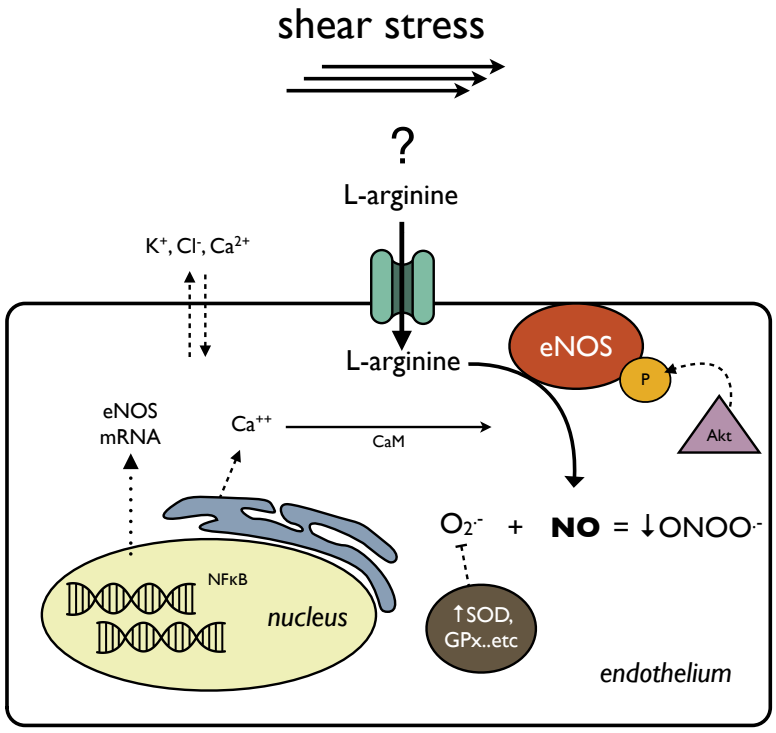

Figure 1.2: **Mechanisms that increase NO production in response to exercise.** Increased blood flow during exercise results in elevated shear stress against the endothelial surface. Acute increases in shear stress result in phosphorylation of eNOS by Akt at serine-1177 resulting in increased eNOS activation [46]. Changes in membrane potential contribute to elevations in intracellular calcium (Ca^{2+}) and can activate eNOS in a Ca^{2+}/Calmodulin (CaM)-dependent manner [8]. Long-term adaptations occur in response to prolonged increases in blood flow that occur with exercise training and result in increased translation of eNOS mRNA via the transcription factor NFκB [39]. Increased expression of antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) scavenge free radicals resulting in improved NO bioavailability [123]. In addition, exercise may potentially improve NO synthesis through increased transport of L-arginine via alterations in CAT-1 expression or transport activity.

1.4.2 Exercise and L-arginine Transport

In addition to the well-described activation of eNOS in response to elevated shear stress, exercise may improve NO production through a mechanism involving increased substrate delivery. Posch et al. (1999) demonstrated that the shear-dependent increase in NO production in porcine aortic endothelial cells (PAECs) was dependent upon an adequate extracellular supply of L-arginine [115]. Importantly, the cellular uptake of L-arginine was increased in a stimulus-dependent manner in response to increasing levels of shear stress [115]. The influence of mechanical stimulation on L-arginine uptake by the endothelium was confirmed in pulmonary arteries exposed to passive stretch [69]. In this study, extracellular L-arginine blunted the increase in tension in response to passive stretch in an NO-dependent manner. Removal of L-arginine from the bathing media or incubation with the L-arginine transport inhibitor, L-Lysine reversed this effect suggesting that passive stretch increased endothelial NO release through a mechanism involving L-arginine transport.

The shear-dependent delivery of L-arginine appears to play an important role in NO synthesis [114, 115] and its impairment may contribute to endothelial dysfunction. Posch et al., (1999) found that shear-dependent L-arginine transport and NO production was impaired in PAECs incubated in glycated LDL isolated from the serum of diabetic patients [114]. The ability of shear stress to augment CAT-1 activity suggests that exercise may be an effective therapy to counteract impairments in L-arginine transport. Indeed, 8 weeks of exercise training has been shown to improve forearm vascular uptake of L-arginine in patients with congestive heart failure [107].

L-arginine supplementation may improve exercise capacity and reduce exercise-induced ROS production, suggesting a synergistic effect of L-arginine and

exercise on vascular function. Chronic supplementation with L-arginine prevented the decline in aerobic exercise capacity in hypercholesterolemic mice through a NO-dependent improvement in exercise-induced hyperemia, however these mice remained sedentary throughout the duration of the supplementation period and were not exercise trained [93]. In addition to improvements in blood flow, L-arginine supplementation has been shown to reduce exercise-induced oxidative stress through increased NOS coupling and scavenging of superoxide through increased peroxynitrite formation [132]. The association between physical activity and L-arginine transport may be most effectively exploited when exercise and L-arginine are used in combination. Dietary supplementation with L-arginine was shown to improve endothelium-dependent dilation to a similar extent as 4 weeks of handgrip exercise training in patients with chronic heart failure; however, the greatest improvement was seen when exercise and L-arginine interventions were treated in combination [60]. The additive nature of L-arginine supplementation with exercise training may have been the result of improved L-arginine transport however there is currently little evidence available to support this hypothesis. Improved L-arginine transport and utilization would be particularly beneficial to patients with CKD and exercise might be a novel therapeutic approach to treat endothelial dysfunction in CKD.

1.4.3 Clinical Implications of Exercise in CKD

Despite the benefits of exercise training on L-arginine transport, NO production, and overall vascular function, the influence of exercise training on these mechanisms in CKD is less established [20]. Low exercise tolerance [74], hypertension [126], insulin resistance and diabetes [161], dyslipidemia [155], and metabolic syndrome [28] are among the many characteristics of patients with CKD.

Based on these symptoms, patients with CKD appear to be ideal candidates for exercise intervention; however, exercise is not typically prescribed [74, 75, 138]. Furthermore, guidelines for the prescription of exercise in patients with CKD are less established [75].

The absence of clinical exercise prescription may stem from concern over the safety of exercise training in patients with CKD [54]. Hyperfiltration of plasma proteins occurs in patients with CKD resulting in proteinuria and has been shown to exacerbate renal damage, resulting in accelerated declines in GFR [122]. Exercise has been shown to cause acute proteinuria in healthy humans [99, 113] and could potentially contribute to further declines in renal damage in patients with already impaired glomerular filtration. Likewise, decreases in renal cortical blood flow [96] and increases in renal sympathetic nerve activity [104] occur during exercise and may exert damaging effects on already ischemic renal tissue in patients with CKD. More recently, exaggerated increases in muscle sympathetic nerve activity (MSNA) and blood pressure in response to handgrip exercise [105] has been demonstrated in patients with CKD.

Despite these seemingly adverse responses to exercise, Fuiano et al., (2004) determined that elevated proteinuria in patients with IgA nephropathy following exercise was transient did not contribute to increases in normal proteinuria associated with renal disease [54]. Likewise, renal impairments were not exacerbated by 12 weeks of treadmill exercise training in rats that underwent renal mass reduction [98]. In fact, glomerular hypertension was reduced in rats that underwent 5/6 nephrectomy after 60 days of exercise training; however, renal function was unaltered [12]. In humans, a single bout of moderate intensity exercise did not alter markers of renal

function in patients with CKD, regardless of disease stage [65]. These studies are in agreement with several other human studies in which the development of renal impairments were either slowed or unaltered by exercise training [18, 49, 82, 108]. Taken together, these studies suggest that exercise is compatible with chronic kidney disease [48].

To date, only a few studies have explored the role of aerobic exercise training in patients with mild to moderate CKD, where the risk of CVD mortality is greatest [68]. In these studies aerobic training interventions consisting of moderate intensity swimming, cycling, and treadmill exercise, collectively resulted in improvements in VO_2 Peak [18, 49, 101], anaerobic threshold [148], peak oxygen pulse and increased peak power output [108]. Despite improvements in aerobic capacity and physical function, the extent to which aerobic exercise reduces the risk for CVD is less conclusive.

While exercise may not be capable of reversing renal damage, it may serve as an adjunct therapy to reduce CVD risk [48]. Evidence from animal studies suggests that 4 weeks of voluntary wheel running prevents the development of vascular dysfunction in 5/6 nephrectomized rats [133] however it remains uncertain if exercise can reverse vascular function once it has already been established. More mechanistic studies are needed to uncover the role of exercise interventions on vascular dysfunction across all stages of CKD.

1.5 Summary

Chronic kidney disease is a major health concern that leads to an accelerated risk of death due to cardiovascular disease. Among the many risk factors for CVD,

impaired endothelial function appears to be an important contributor in patients with CKD. There is an urgent need to understand the mechanisms by which the endothelium becomes impaired in CKD in order to design more effective strategies for reducing CVD risk. Elevated oxidative stress, L-arginine deficiency, and increased levels of ADMA all appear to play a role in the pathogenesis of endothelial dysfunction in CKD. Studies of vascular function across all stages of CKD suggest that endothelial dysfunction may become less reversible with advancing kidney disease [91]. Recent evidence suggests that exercise may serve as a novel adjunct therapy to reverse vascular impairments in CKD; however, few studies have been conducted. Our goal was to explore the role of these mechanisms in the pathogenesis of endothelial dysfunction in CKD and to explore the potential ability of increased physical activity to improve endothelial function.

Chapter 2

IMPAIRED L-ARGININE UPTAKE BUT NOT ARGINASE CONTRIBUTES TO ENDOTHELIAL DYSFUNCTION IN RATS WITH CHRONIC KIDNEY DISEASE[1]

2.1 Introduction

Chronic kidney disease (CKD) affects 26 million people in the United States [35] and is a rapidly growing public health concern [127]. The risk of developing cardiovascular disease (CVD) is very high in patients with CKD, and persists even in the absence of traditional cardiovascular risk factors, making patients with CKD more likely to die of a cardiovascular event than progress to end-stage renal disease (ESRD) [126]. The endothelium is an important regulator of vascular tone via the release of vasodilators including nitric oxide (NO). Reduced NO production or bioavailability is characteristic of endothelial dysfunction and plays an early role in the development of hypertension and atherosclerosis [38, 136]. Endothelial dysfunction has been shown to occur in animals and humans with chronic kidney disease [4, 11, 36, 63, 164] and may play an important role in the increased cardiovascular risk in CKD [45].

The synthesis of endothelial derived NO occurs via endothelial nitric oxide synthase (eNOS) from the amino acid substrate L-arginine [97]. Treatment with exogenous L-arginine has been shown to improve vascular function in aging [15] and

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[1] Reprinted with permission from: Martens CR, Kuczmarski JM, Lennon-Edwards S, Edwards DG. Impaired L-arginine uptake but not arginase contributes to endothelial dysfunction in rats with chronic kidney disease. *J Cardiovasc Pharmacol*. 2013. DOI: 10.1097/FJC.0000000000000022

hypercholesterolemia [31, 33] suggesting that decreased substrate availability contributes to endothelial dysfunction.

Among the mechanisms that regulate L-arginine availability, the arginase pathway has gained considerable attention in the literature. Arginase catalyzes the conversion of L-arginine to urea and ornithine during the urea cycle, and is thus capable of reducing NO production by competing with eNOS for substrate [47]. Several studies have demonstrated in both human [66, 67] and animal [7, 13, 162] models of aging and hypertension that arginase inhibition restores endothelium-dependent relaxation through improved NO production. In addition, long-term arginase inhibition has been shown to delay the progression of CKD in animals that underwent a 5/6 ablation/infarction surgery [124]; however, it is unclear whether arginase is a suitable therapeutic target for improving vascular function in CKD.

The purpose of this study was to determine if arginase contributes to endothelial dysfunction in rats with CKD. We hypothesized that CKD would result in increased arginase protein content and activity and would be associated with impaired endothelial function that would be reversed by arginase inhibition.

2.2 Methods

2.2.1 Ethical Approval

All surgical procedures and experiments were approved by the University of Delaware Institutional Animal Care and Use Committee and were conducted in accordance with the Public Health Service Policy regarding the Humane Care and Use of Laboratory Animals.

2.2.2 Animal Model of CKD

CKD was induced in 12-week old male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) by 5/6 ablation infarction (AI) surgery [50]. Surgery was performed under isoflurane (1.5-5.0%) using sterile technique and consisted of ligating 2/3 of the left renal artery branches, followed by complete removal of the right kidney. Animals were compared to a sham-control group that underwent a similar surgery where no renal tissue was infarcted or removed. Animals were individually housed in standard caging and kept on a 12-hour light/dark cycle. All animals were fed a standard rat chow diet and given free access to food and water.

2.2.3 Development of Kidney Disease

Animals were monitored over the course of 8 weeks for the development of kidney disease. Based on previous work by Chen & Baylis (2010), this time frame is sufficient for animals to develop moderate to severe kidney disease [27]. We assessed renal function by measuring serum creatinine, and blood urea nitrogen (BUN) at baseline, 3, and 7 weeks after surgery and urinary excretion of protein at baseline and again every 2 weeks until the end of the study. Serum uric acid was also measured. Urine protein excretion was determined by performing timed, 16-hour urine collections in a metabolic cage. Animals were allowed free access to water but restricted from food for the duration of the collection. Urine volumes were recorded and samples were stored at -80°C for later analysis of urinary protein using the Bradford method ([19]; Biorad; #500-0203) and urinary excretion of nitrate/nitrite (NOx) as an index of systemic nitric oxide production (Cayman Chemical; #780001). Blood samples were obtained from the tail vein, and centrifuged at 3,000 rpm for 10 minutes. Serum was stored at -80°C for later assay of creatinine, BUN, and uric acid

concentrations. All serum samples were analyzed using a Siemens Dimension RxL Max clinical chemistry system. Serum creatinine was determined based on a Jaffe reaction between creatinine and picrate in which the reaction product was measured at an absorbance of 510 nm. BUN was determined from the conversion of urea to L-glutamate by urease and glutamate dehydrogenase where change in absorbance at 340 nm is directly related to BUN concentration. Uric acid was determined from the change in absorbance at 293 nm from the conversion of uric acid to allantoin by the enzyme uricase.

2.2.4 Renal Pathology

Kidney damage was assessed from fixed kidney sections by calculating the glomerulosclerosis index (GSI) as described in detail elsewhere [98, 117]. Upon sacrifice, the left kidney was dissected and a transverse section was prepared and fixed in 10% formalin. Samples were sent to the Comparative Pathology Laboratory at the University of Delaware for staining and determination of GSI. Fixed tissue was paraffin embedded, prepared into 4 μm sections and stained with periodic acid schiff (PAS; Sigma-Aldrich #395B-1KT). Staining was confirmed using positive control tissue (kidney) from another source. All tissues were microscopically evaluated at 400x magnification by a single ACVP-certified veterinary anatomic pathologist in a blinded fashion. Glomeruli were scored on a scale from 0-4 with 0=healthy glomeruli, 1<25% damage, 2=25-50% damage, 3=51-74% damage, and 4>75% damage. GSI was calculated using the following equation: [(# of 1) + 2(# of 2) + 3(# of 3) + 4(# of 4) / total number of glomeruli observed].

2.2.5 Sacrifice and Preparation of Tissue

Animals were anesthetized with isoflurane (1.5-5.0%) prior to sacrifice and a 1.4 french pressure transducer (Millar Instruments, Houston, TX) was inserted through the femoral artery into the thoracic aorta to obtain blood pressure. Blood pressure recordings were obtained after 10 minutes of equilibration on a heating pad. Animals were sacrificed by exsanguination via removal of the heart and the thoracic aorta was dissected and placed in ice-cold physiological salt solution (PSS; NaCl, 118.99 mmol; KCl, 4.69 mmol; $CaCl_2$-$2H_2O$, 2.50 mmol; $MgSO_4$-$7H_2O$, 1.17 mmol; $KH2PO_4$, 1.18 mmol; EDTA, 0.03 mmol; Glucose, 1.091 g/L; $NaHCO_3$ 2.100 g/L; pH, 7.4). Aortas were cut into 3mm ring sections for assessment of vascular function, or L-arginine transport as described below. The remaining tissue was quickly frozen in PSS using liquid nitrogen and stored at -80°C for later assessment of CAT-1, eNOS, and arginase protein expression.

2.2.6 Assessment of Vascular Function

Vascular relaxation was assessed *in vitro* by performing isometric ring studies using aortic ring segments. Rings were prepared as described above and mounted on wire force transducers (DMT 610M, Danish Myotechnology) within individual organ baths containing PSS at 37°C and pH 7.4. Vessels were oxygenated with carbogen gas (5% CO_2, 95% O_2) and set to a resting tension of 20mN. Following an equilibration period and tests of viability, rings were treated in the presence of 10μM of the NOS inhibitor L-NAME (Sigma-Aldrich, N5751) to determine if impaired endothelium-dependent relaxation was NO-mediated. Rings were preconstricted with phenylephrine (PE; $3x10^{-7}$M) and relaxed with incrementing doses of acetylcholine (Ach; 10^{-9} – 10^{-5}M) to assess endothelium-dependent relaxation. Using the same

methods, a separate set of rings were pre-treated with specific drugs, and endothelial function was assessed to answer the following questions:

2.2.6.1 Arginase Inhibition

To determine if arginase contributes to endothelial dysfunction by reducing substrate availability for eNOS, aortic rings were incubated with 10μM of the arginase inhibitor S-(2-Boronoethyl)-L-cysteine hydrochloride (BEC; Calbiochem, 197900) alone or in combination with 100μM L-arginine prior to determination of endothelium-dependent relaxation. The dose of L-arginine was selected so that it satisfied the Km concentration of both eNOS and the L-arginine transporter CAT-1 [13]. Importantly, this dose does not approach the dissociation constant of L-arginine for arginase (1mM) [30] ensuring that L-arginine did not interfere with the ability of BEC to inhibit arginase activity. The dose of BEC administered was chosen as it is ~5 times the dissociation constant of BEC for arginase [30] and has previously been shown to contribute to endothelium-dependent relaxation of aortic rings [13, 70]. To confirm the efficacy of this dose, we performed additional experiments in which arginase activity was assessed in aortic homogenates in the presence or absence of 10μM BEC (data not shown). Six animals were used in each group.

2.2.6.2 Oxidative Stress

To determine if the effects of L-arginine and/or arginase inhibition were masked by underlying oxidative stress, rings were pre-treated with 30 μM of the superoxide scavenger Tempol (Sigma-Aldrich, 176141) alone or in combination with either L-arginine or L-arginine + BEC. The dose of Tempol was based on work previously reported by Viswanad et al. [157]. Following a 30-minute incubation

period, endothelium-dependent relaxation was assessed as described above. Seven animals were used in each group.

2.2.7 Arginase Activity

Aortic tissue samples were homogenized and centrifuged using the method described above. Supernatant was prepared in a 1:9 dilution and used to assess arginase activity from the production of urea using an arginase assay kit (QuantiChrom DARG-200, BioAssay Systems). Urea concentration was determined colorimetrically by reading the absorbance at 520 nm. Data are presented in arginase activity units (AAU) per mg protein. Protein concentrations were determined using the Bradford protein assay ([19]; Biorad; #500-0203). One AAU converts one μmole L-arginine to urea and ornithine per minute. Values were corrected for pre-existing urea in the sample to control for elevated levels of urea associated with CKD.

2.2.8 Western Blot

Protein expression arginase I & II, CAT-1, eNOS, and phosphorylated eNOS (ser-1177) were assessed by Western Blot. Aortic tissue samples were homogenized in lysis buffer (1x PBS at pH 7.4 containing: 0.1% SDS, 0.01% Triton X-100, protease inhibitors (Sigma Aldrich, #P8340). Tissue homogenates were centrifuged at 10,000g for 10 min at 4°C and supernatant was extracted and prepared for analysis. Protein concentration was determined using the Bradford method ([19]; Biorad; #500-0203). Samples were loaded into a 10% Tris-HCl gel (Bio-Rad 161-1175) and electrophoresed for 60 minutes at 100V. Gels were transferred to a nitrocellulose membrane and immunoblotted with the primary antibody for the protein of interest (arginase I (1:1000; Abcam ab60176), arginase II (1:2000; Abcam ab6885), CAT-1

(1:200; Santa Cruz sc-66825), eNOS (1:1000; BD 610297), eNOS-1177 (1:500; BD 612393)), followed by the appropriate recommended secondary antibody. Membranes were incubated with developing reagents (GE Healthcare; RPN2109). Data are presented as intensity relative to β-actin (1:2000; Santa Cruz Biotechnology sc-81178). Western blots were quantitated using Image J software from NIH.

2.2.9 L-Arginine Transport

L-arginine transport was assessed in aortic rings using an established *in vitro* technique [72, 128, 129]. Aortic rings were incubated for 1 minute at 37°C in 0.5 M HEPES buffer containing 1mM L-arginine and 10μCi L-[^{3}H]arginine. A separate set of rings were incubated in buffer containing no L-[^{3}H]arginine as a control. One minute was selected because it falls within the linear portion of the L-arginine uptake curve [128]. After one minute, rings were washed with ice-cold PSS and homogenized in lysis buffer. Tissue homogenates were analyzed for radioactivity (disintegrations per minute) by liquid-scintillation counting using a Packard Tri-Carb 2900-TR liquid scintillation counter.

2.2.10 Statistical Analysis

Urine protein and NOx excretion, BUN, serum creatinine, and uric acid were analyzed with a 2-way repeated-measures ANOVA and between group differences were assessed with a Bonferroni post-hoc test. Heart mass/ tibia length ratio, western blot, and arginase activity data were analyzed with an unpaired, two-tailed t-test. Dose response curves were generated using GraphPad Prism 5.0 software and normalized to % relaxation with the preconstriction value set at 0%. $LogEC_{50}$ and maximal relaxation (E_{max}) were compared between groups for each treatment using an

unpaired, two-tailed t-test. The alpha level was set at 0.05 and all data are presented as means ± SEM.

2.3 Results

Table 2.1 contains renal function measurements made over the course of 8 weeks following surgery. Urine protein excretion was significantly elevated in AI animals compared to sham animals after 5 weeks while serum creatinine, and BUN was significantly elevated in AI animals after 3 weeks. Uric acid levels did not change between groups at any time point.

Glomerulosclerosis (GSI) data are presented in Figure 2.1. GSI scores were significantly greater in AI animals and are indicative of severe renal damage, with little to no damage observed in sham animals. Table 2.2 contains cardiovascular measurements including mean arterial pressure (MAP), systolic blood pressure (SBP), diastolic blood pressure (DBP), pulse pressure (PP), heart mass/tibia length ratio, and urinary excretion of nitrate and nitrite (NOx). MAP, SBP, DBP, and PP were elevated in AI animals compared to sham but did not reach significance (Table 2.2). There was a slight, though not significant increase in heart mass ($p=0.101$) and heart mass/tibia length ratio ($P = 0.154$) in the AI group compared to sham. Urinary NOx excretion declined over time in both groups however the decline occurred to a greater extent in AI animals and was significantly attenuated from sham animals at 4 and 7 weeks post surgery ($p<0.05$).

Table 2.1 Body Mass and Renal Function Measurements

	Sham (n=5)	**AI (n=5)**
Body Mass (BM) (g)	409.8 ± 11.94	376.3 ± 24.63 *
Urine Protein Excretion (mg•24 hr^{-1}•100g^{-1}BM)		
Baseline	5.4 ± 1.66	4.9 ± 1.04
Week 1	3.5 ± 1.43	8.3 ± 2.16
Week 3	4.6 ± 1.03	23.6 ± 11.9
Week 5	4.7 ± 1.45	36.3 ± 9.19 *
Week 7	4.5 ± 1.08	39.8 ± 15.6 **
Serum Creatinine (mg/dL)		
Baseline	1.1 ± 0.5	0.8 ± 0.3
Week 3	0.9 ± 0.4	1.3 ± 0.6
Week 7	1.0 ± 0.4	1.6 ± 0.7 *
BUN (mg/dL)		
Baseline	35 ± 16	26 ± 12
Week 3	36 ± 16	65 ± 29 *
Week 7	32 ± 14	86 ± 39 *
Uric Acid (mg/dL)		
Baseline	1.7 ± 0.8	1.3 ± 0.6
Week 3	1.9 ± 0.9	1.5 ± 0.7
Week 7	1.6 ± 0.7	1.5 ± 0.7

Values are mean ± SEM. n, number of animals; AI, ablation/infarction; BUN, blood urea nitrogen. * $P < 0.05$ vs Sham, ** $P < 0.01$ vs Sham

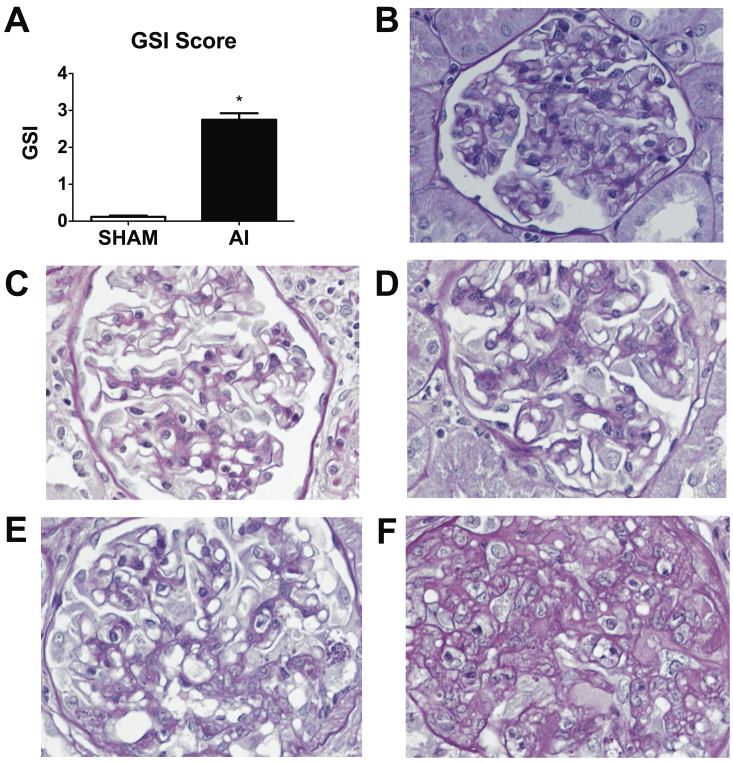

Figure 2.1: **Glomerulosclerosis Index (GSI)** in sham vs. AI animals (A). Glomeruli at 400x magnification exhibiting Grade 0 (B), Grade 1(C), Grade 2 (D), Grade 3(E), and Grade 4 (F) glomerulosclerosis scores based upon extent of hyalinosis and sclerosis within glomerular tuft. Grade 0 indicates a normal glomerulus and Grade 4 indicates total obliteration and collapse of the glomerular tuft. * $p < 0.0001$ between groups. N = 5 in each group.

Table 2.2 Cardiovascular Measurements

	Sham (N=5)	**AI (N=5)**
Mean Arterial Pressure (mmHg)	102.29± 8.55	115.60 ± 5.20
Systolic Blood Pressure (mmHg)	125.09 ± 4.48	134.22 ± 11.12
Diastolic Blood Pressure (mmHg)	81.49 ± 5.47	93.46 ± 8.79
Pulse Pressure (mmHg)	43.60 ± 1.52	40.76 ± 7.4
Heart mass (g)	1.577 ± 0.094	1.770 ± 0.108
Heart mass/tibia length (g/cm)	0.4207 ± 0.021	0.4528 ± 0.022
Urinary NOx Excretion (nmol/100g/24 hr)		
Baseline	1454 ± 142	1717 ± 164
Week 3	1199 ± 157	528 ± 119 *
Week 7	902 ± 91	373 ± 102 *

Values are mean ± SEM. n, number of animals; AI, ablation/infarction; NOx, urinary nitrates/nitrites. * $P < 0.05$ vs. Sham

2.3.1 Isometric Ring Studies

The endothelium-dependent relaxation response to acetylcholine was significantly attenuated in both sham and AI animals when rings were treated with the NOS inhibitor L-NAME (Figure 2.2) indicating that EDR was primarily mediated by a reduction in nitric oxide.

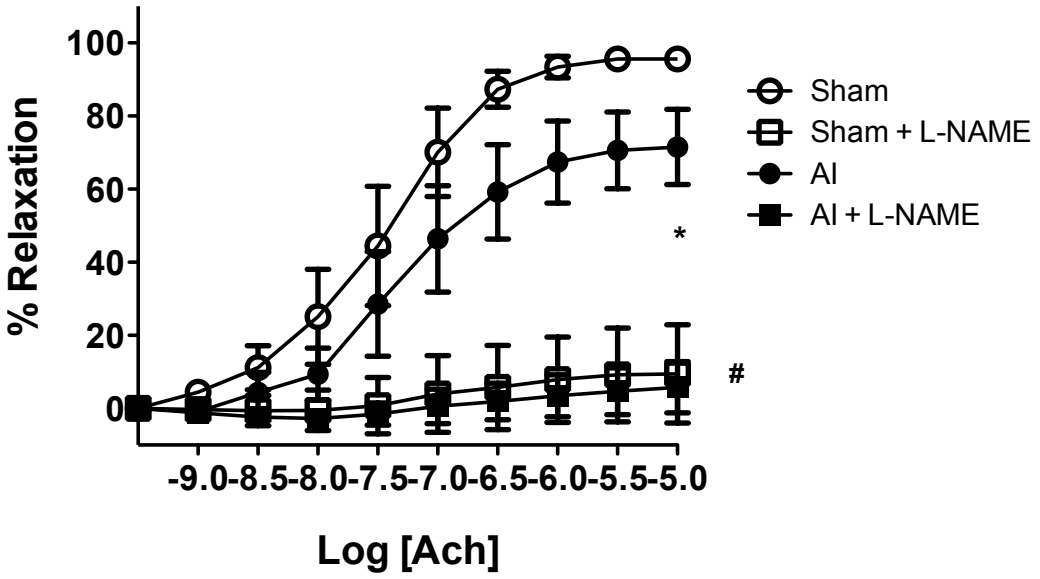

Figure 2.2: **Endothelium-dependent relaxation (EDR).** Dose response of aortic rings treated with acetylcholine (Ach) under control conditions, and treated with the NOS inhibitor N (G)-nitro-L-arginine methyl ester (L-NAME). Maximal EDR response was different in control rings between groups, $p < 0.05$. # Maximal EDR response and LogEC50 of L-NAME treated rings were significantly different from respective controls, $p < 0.05$. n=6 in each group.

2.3.1.1 Arginase Inhibition

Endothelium-dependent relaxation was impaired in untreated control rings of AI animals. As indicated in Figure 2.3A, rings from AI animals required a larger dose of Ach before relaxation occurred resulting in a rightward shift in the $LogEC_{50}$ ($p<0.001$) and a blunted maximal response (E_{max}; $p=0.001$). EDR remained significantly impaired in AI animals following treatment with L-arginine (Figure 2.3B; $LogEC_{50}$ $p<0.01$, E_{max} $p<0.01$). Arginase inhibition with BEC alone (Figure 2.3C; $LogEC_{50}$ $p=0.01$, E_{max} $p<0.01$) did not improve EDR and Log EC_{50} remained significantly impaired in rings co-treated with L-arginine and BEC (Figure 2.3D; $LogEC_{50}$ $p=0.01$, E_{max} $p<0.12$).

2.3.1.2 Oxidative Stress

As observed in our arginase studies, endothelium-dependent relaxation was significantly impaired in control rings of AI animals (Figure 2.4A; LogEC50 $p = 0.71$, Emax $p<0.05$). Impaired EDR persisted following scavenging of superoxide with Tempol (Figure 2.4B; LogEC50 $p=0.15$, Emax $p < 0.05$). Co-treatment of rings with Tempol in the presence of L-arginine (Figure 2.4C; LogEC50 $p < 0.01$, Emax $p < 0.01$) and L-arginine + BEC (Figure 2.4D; LogEC50 $p = 0.11$, Emax $p < 0.05$) did not improve EDR.

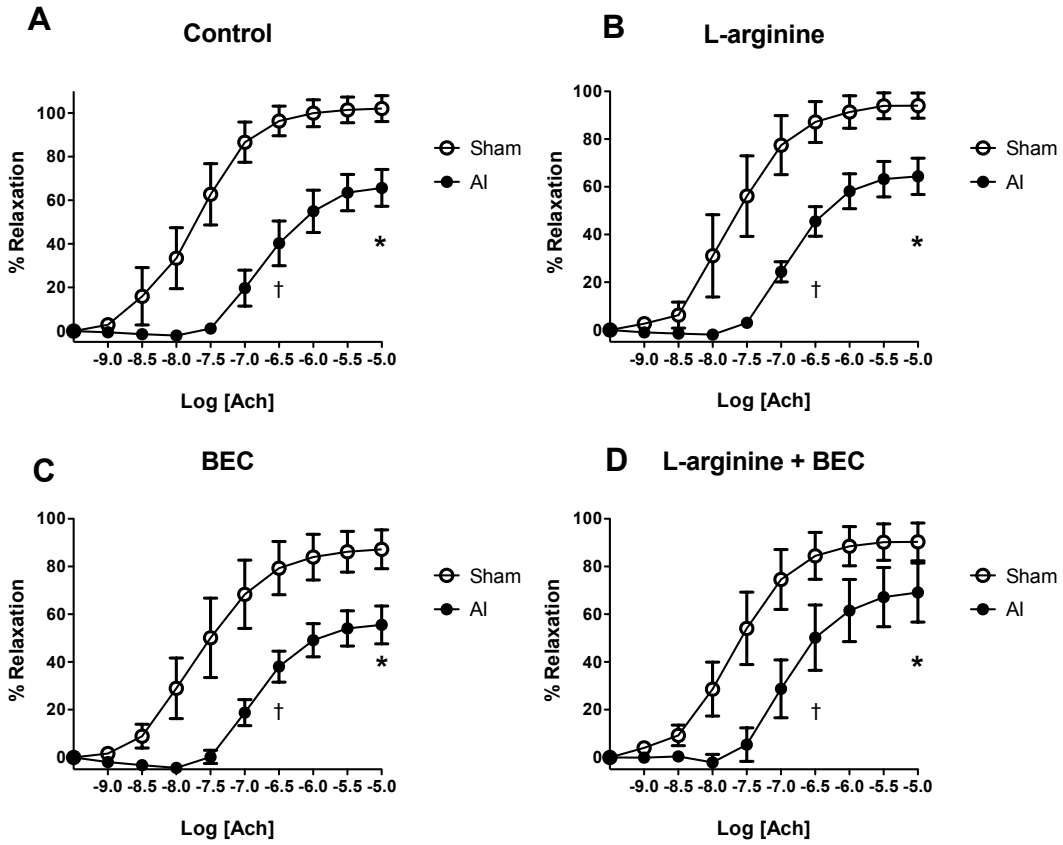

Figure 2.3: **Effect of L-arginine and/or arginase inhibition on EDR** Endothelium-dependent relaxation (EDR) response of aortic rings to acetylcholine (Ach) under control conditions (A), treated with L-arginine (B), with arginase inhibition using BEC (C) and treated with L-arginine and BEC (D). * Maximal effect different between groups, $p < 0.05$. † LogEC50 different between groups, $p < 0.05$, $n = 6$ in each group.

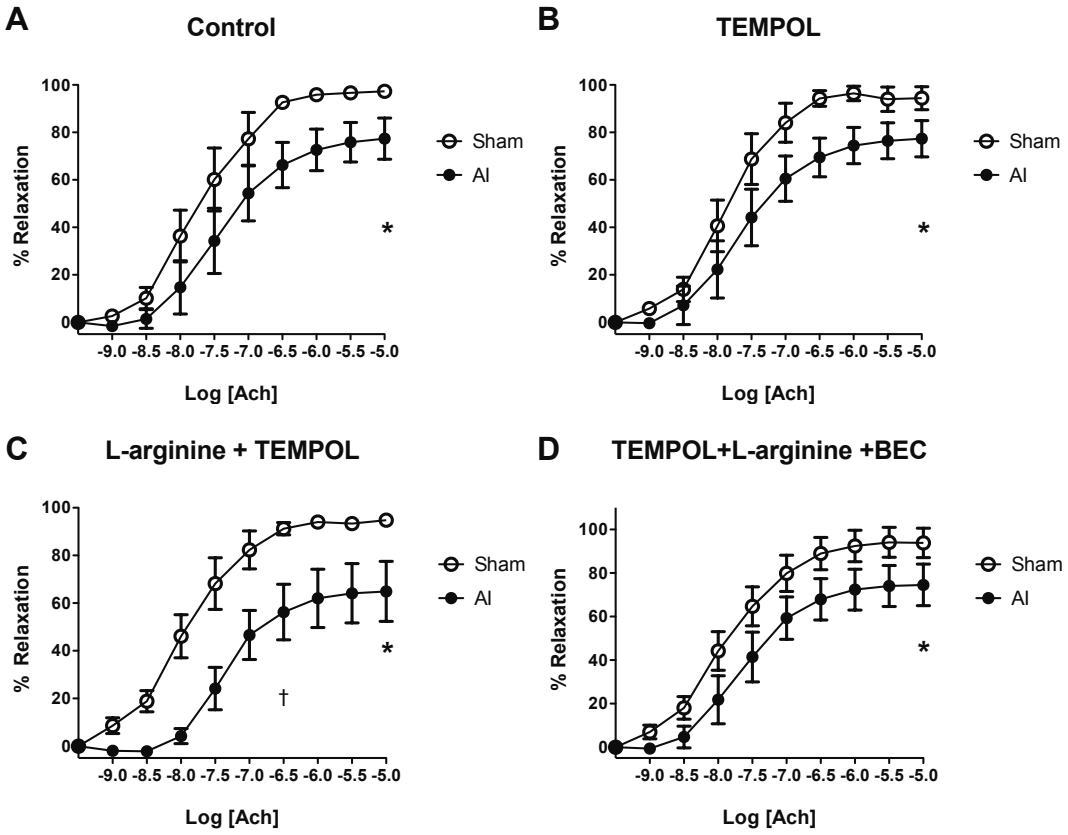

Figure 2.4: **Effect of Tempol, L-arginine, and arginase inhibition on EDR** Endothelium-dependent relaxation (EDR) response of aortic rings to acetylcholine (Ach) under control conditions (A), treated with Tempol (B), Tempol + L-arginine (C) and Tempol + L-arginine + BEC (D). * Maximal effect different between groups, $p < 0.05$. † LogEC50 different between groups, $p < 0.05$. $n = 7$ in each group.

2.3.2 Tissue Analysis

There was no significant difference between AI and Sham expression of arginase I (p=0.098; Figure 2.5A) or arginase II (p=0.645; Figure 2.5B) relative to β-actin. There was also no significant difference in arginase activity between AI and Sham (p=0.823) (Figure 2.5C).

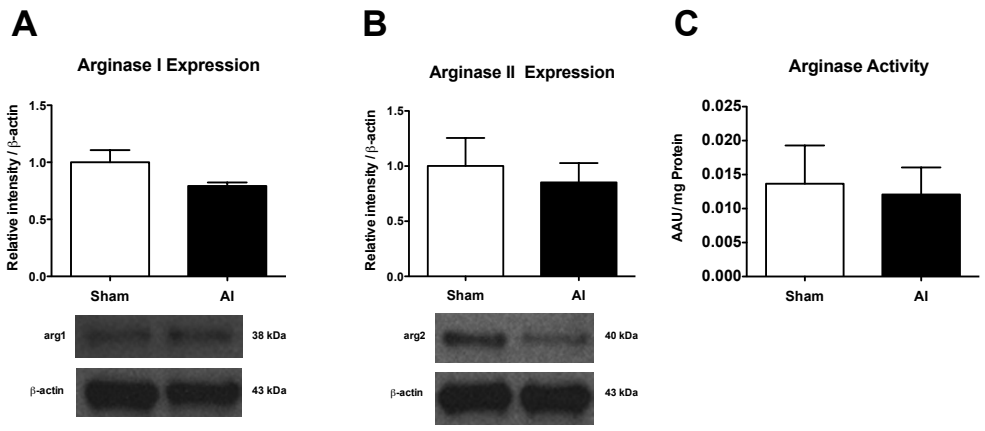

Figure 2.5: **Arginase protein expression and enzymatic activity** Arginase I (A) and Arginase II (B) intensity normalized to β-actin measured by western blot. (C) Aortic arginase activity expressed in arginase activity units (U) relative to protein concentration (U/mg protein). Not significant. n = 5 in each group.

L-arginine transport was attenuated in AI animals as indicated by a reduction in aortic uptake of L-[^{3}H]arginine compared to sham controls (p=0.03; Figure 2.6A). This was accompanied by a 63% reduction in aortic protein expression of CAT-1 (p = 0.001; Figure 2.6B). eNOS expression declined in AI animals by ~30% however this result was not significant (p = 0.183; Figure 2.7A). No difference in eNOS phosphorylation at ser-1177 was observed between groups (p = 0.207; Figure 2.7B).

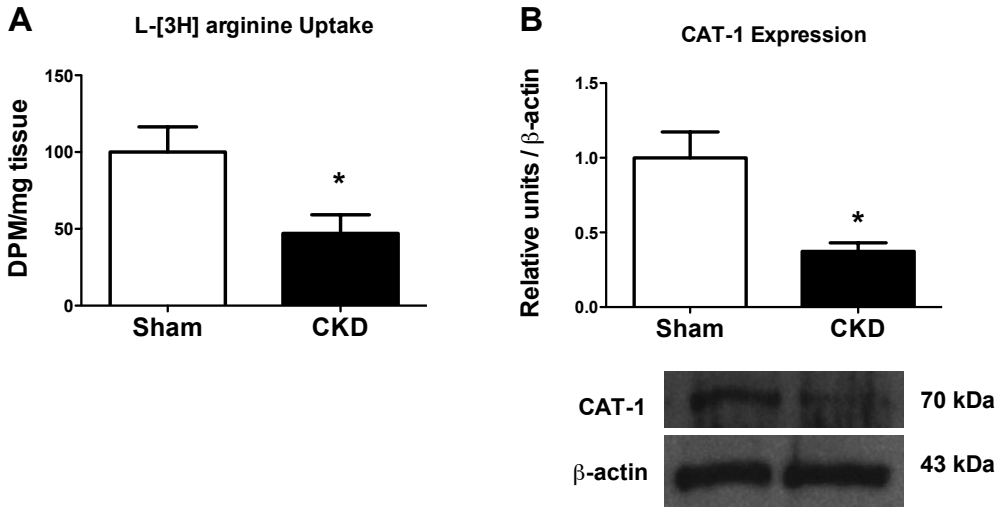

Figure 2.6: **L-arginine uptake and CAT-1 protein expression** L-[^{3}H] arginine uptake, disintegrations per minute (DPM) normalized to wet tissue mass. Sham n=6, AI n=5 (B) CAT-1 intensity normalized to β-actin, measured by western blot. Sham n = 13, AI n = 11. * $p<0.05$.

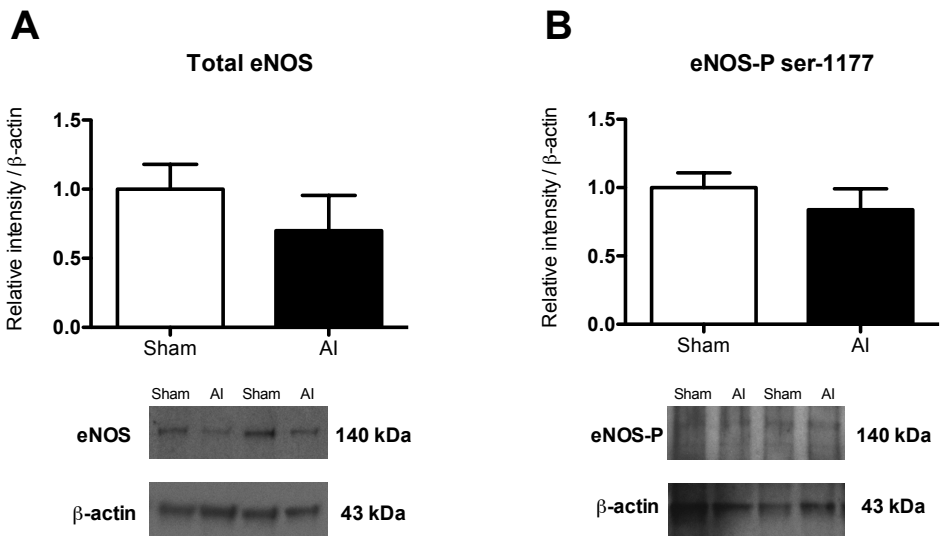

Figure 2.7: **Total and phosphorylated eNOS protein expression** (A) Total eNOS intensity and (B) phosphorylated eNOS (ser-1177) intensity normalized to β-actin, measured by western blot. n= 5 in each group. p = 0.183

2.4 Discussion

The primary finding of this study is that arginase inhibition, alone or in combination with L-arginine treatment did not restore endothelium-dependent relaxation (EDR) in aortic rings of rats 8 weeks after 5/6 A/I induced CKD. Furthermore, aortic arginase expression and activity were not different from sham animals suggesting that arginase does not contribute to vascular dysfunction in moderate to severe CKD. We also found these results to be independent of oxidative stress as co-incubation of these drugs with the superoxide dismutase mimetic Tempol, did not restore EDR. Instead, endothelial dysfunction in late-stage CKD may be linked to a reduction in substrate availability for eNOS through a mechanism involving impaired L-arginine transport.

Arginase contributes to L-arginine metabolism through the formation of urea and ornithine during the urea cycle, and is capable of reducing NO production by competing with eNOS for substrate [47]. Supplementation with a manganese (Mn^{2+})-free diet for 8 weeks was shown to suppress arginase activity in the liver and kidney of rats that underwent 5/6 A/I surgery [124]. This reduction delayed the progression of renal failure and was associated with an increase in urinary NOx excretion that was most likely due to improved substrate availability for eNOS. While this chronic suppression of arginase activity was shown to delay the progression of renal failure and improve NO production, it is uncertain whether this treatment would have been effective if initiated later in the development of CKD. In addition, the authors were unable to separate the effects of the treatment on renal and hepatic tissue from that on the peripheral vasculature. Our data provide important and novel insight as we have chosen to study the specific role of arginase in the vasculature of animals that have already progressed to a more severe level of CKD.

Endothelial-derived NO production from the peripheral vasculature including the aorta and conduit vessels plays an important role in the maintenance of vascular homeostasis through improved vasodilation [97] and arterial compliance [81]. The two known isoforms of arginase have been identified in the vasculature [70] and have been shown to contribute to endothelial dysfunction through NO-mediated mechanisms in both human [66, 67], and animal models [7, 13, 162] of aging, hypertension, and hypercholesterolemmia. Antisense knockdown of arginase I significantly increased eNOS activity and NO production in aged rats [162] while inhibition of arginase with 10μM BEC improved the relaxation response to 100 μM L-arginine in aortic rings of old rats to a similar level as their younger counterparts [13]. The majority of data implicating arginase in vascular dysfunction have come from aging and other models, and the therapeutic potential of arginase as a possible treatment for endothelial dysfunction in CKD is less clear. Recently, Chen et al. (2012) demonstrated no increase in aortic arginase expression in rats treated with a high dose of puromycin aminonucleoside (PAN) to induce severe CKD [26]. While this model differs from the 5/6 A/I model, it is similar to our own finding that arginase does not contribute to endothelial dysfunction in late-stage CKD. Interestingly, rats treated with a low dose of PAN as a model of more moderate CKD demonstrated a significant increase in arginase expression suggesting that arginase may be important in the early development of endothelial dysfunction; however, the role of arginase on EDR in mild to moderate CKD has not yet been investigated.

The influence of arginase on vascular function in more severe CKD may be suppressed through a negative feedback mechanism. Arginase is a key contributor to the production of urea, a byproduct of protein metabolism and a “uremic toxin” that is

retained in the blood in patients with chronic kidney disease. The increase in circulating urea associated with CKD may decrease the activity of arginase in a negative feedback manner such that the contribution of arginase to L-arginine metabolism is reduced under uremic conditions. This type of inhibition has been demonstrated in liver homogenates from rats 6 weeks following 5/6 nephrectomy [100] and may explain why arginase inhibition did not restore endothelial function in our own animals. This mechanism may also explain the findings of Chen et al. (2012) who found no change in arginase in rats treated with a high dose of PAN to induce severe CKD [26]. Additionally, elevated levels of uric acid have been shown to increase arginase activity and impair EDR *in vitro* [170]; however, we found no difference in serum levels of uric acid between 5/6 A/I and sham animals.

In addition to reduced substrate availability, oxidative stress is another potential contributor to endothelial-dysfunction through its ability to reduce NO production and bioavailability. Angiotensin II is elevated in CKD and has been shown to activate the NAD(P)H oxidases resulting in increased production of the powerful free radical superoxide [43, 77, 154]. To determine if increased superoxide production played a role in the impaired EDR response to acetylcholine, we treated rings with the superoxide scavenger, Tempol. we observed no effect of Tempol alone on EDR suggesting that oxidative stress is not the primary mechanism behind impaired EDR in this model of CKD. More importantly, Tempol was unable to restore EDR when treated in combination with L-arginine, or when combined with both L-arginine and BEC. These findings demonstrate that any effect of arginase inhibition and/or L-arginine were not simply masked by underlying superoxide production and suggest that some other mechanism must be present to impair NO production.

Surprisingly, L-arginine itself was ineffective suggesting that other mechanisms contribute to endothelial dysfunction and impaired substrate availability. While the intracellular concentration of L-arginine has been shown to exceed the Km of eNOS for L-arginine [61], exogenous treatment with L-arginine has been largely effective at restoring endothelial function in aging [15] and hypercholesterolemia [31, 33]. This phenomenon, referred to as the "arginine paradox" [16, 61] suggests that NO production relies primarily on extracellular L-arginine rather than intracellular stores. Indeed, transport of extracellular L-arginine into the endothelium through CAT-1 stimulates NO production [169]. CAT-1 has been shown to form a caveolar complex with eNOS [87] suggesting a system by which facilitated transport of extracellular L-arginine is coupled to NO production. This system seems like an attractive target to treat endothelial dysfunction in CKD since the synthesis and release of L-arginine by the kidneys is impaired with kidney damage [10, 27]. Unfortunately, L-arginine treatment in CKD has been met with mixed results [11, 36, 164]. Chronic treatment with L-arginine for 9 weeks prevented the development of endothelial dysfunction in 5/6 nephrectomized rats [164]; however, L-arginine did not improve endothelial function in adults or children with renal failure [11, 36]. These findings suggest that while L-arginine may prevent the development of vascular dysfunction early in the progression of CKD, it is less effective once CKD has already been established. Recent evidence supports this conclusion by suggesting a link between high levels of uremic toxins and impaired L-arginine transport [10, 119]. Interestingly, impaired L-arginine delivery to the endothelium occurs in CKD despite a maintenance of normal plasma L-arginine concentrations [10]. This has been suggested to result from both compensatory increases in L-arginine synthesis by other

tissues as well as "trapping" of L-arginine within the vasculature due to the reduced transport activity [10].

The transport of L-arginine into the endothelium occurs through the cationic amino acid transporter, CAT-1 in a sodium and pH independent manner that is characteristic of the more classically defined system y+ transporter [32]. Endothelial uptake of L-arginine through CAT-1 has been shown to be inhibited in endothelial cells cultured in uremic plasma and in cells cultured in a synthetic solution containing uremic levels of urea [163], suggesting a role of uremic toxins in L-arginine transport inhibition. These findings have been confirmed previously in uremic rats [128] and our results are consistent demonstrating a significant attenuation in L-arginine transport accompanied by a reduction in CAT-1 expression. Though not significant, eNOS expression was attenuated by ~30% AI animals compared to the sham group. It is therefore possible that NO synthesis is dependent upon both adequate substrate delivery as well as eNOS expression. Future studies should explore the co-localization and interaction between eNOS and CAT-1 to confirm this hypothesis. Importantly, these findings suggest that decreased L-arginine transport may contribute to functional impairments in vascular relaxation in CKD.

In conclusion, L-arginine treatment and arginase inhibition, alone or in combination, does not restore aortic endothelium-dependent relaxation in a model of moderate to severe CKD. Our data in an animal model of CKD support observations in cell-culture studies suggesting that L-arginine transport [158, 163] and arginase activity [100], are limited by uremic levels of urea. Together, these data suggest that arginase inhibition may not be the most effective therapy to improve cardiovascular health in patients with CKD; however, future work is necessary to determine the role

of arginase and L-arginine in endothelial function at earlier stages of CKD when the uremic burden is not as severe.

Finally, impaired vascular uptake of L-arginine may play a key role in the pathogenesis of endothelial dysfunction and should be explored as a potential therapeutic target. Future studies should focus on the specific mechanisms by which CAT-1 protein and mRNA expression are regulated. Interestingly, CAT-1 mRNA has been shown to increase in the vasculature of 5/6 nephrectomized rats despite a decline in protein abundance [128], suggesting that CAT-1 is regulated by post-translational modifications; however, the mechanisms remain unclear. Additionally, the kinetics of L-arginine uptake should be more thoroughly explored to help guide the design of interventions aimed at improving L-arginine transport in CKD.

Chapter 3

VOLUNTARY WHEEL RUNNING AUGMENTS AORTIC L-ARGININE TRANSPORT AND ENDOTHELIAL FUNCTION IN RATS WITH CHRONIC KIDNEY DISEASE

3.1 Introduction

Endothelial dysfunction contributes to the development of cardiovascular disease (CVD) in patients with chronic kidney disease (CKD) and is primarily associated with a decrease in nitric oxide production and impaired endothelium-dependent relaxation [89]. The decline in endothelial function precedes the development of atherosclerosis [38, 136] and has been extensively studied as a potential therapeutic target to treat CVD; however, the specific mechanisms of endothelial dysfunction in CKD have not been fully elucidated. Patients with CKD are more likely to die of CVD than progress to end-stage renal disease [73, 126]; therefore, novel treatments to improve endothelial function in CKD are needed to reduce CVD-related mortality in CKD.

Insufficient availability of the NO precursor, L-arginine likely contributes to reduced NO synthesis in CKD [10]. Interestingly, the use of L-arginine in studies of endothelial dysfunction in late-stage CKD has produced mixed results [11, 36] unlike other conditions where it has been largely effective [15, 31, 33, 66]. Evidence from cell culture studies suggests that urea and other uremic toxins inhibit L-arginine uptake into endothelial cells [158, 163] by acting on the L-arginine transporter, cationic amino acid transporter 1 (CAT-1). We have recently shown that

endothelium-dependent relaxation (EDR) was not improved *in vitro* by an exogenous dose of L-arginine in an animal model of moderate to severe CKD [90]. This finding was associated with reduced CAT-1 protein expression and L-arginine transport in isolated aortic rings [90] and may provide a unique explanation for why treatment with L-arginine has been ineffective in CKD. Therapies aimed at improving the utilization of L-arginine would be particularly beneficial for the restoration of endothelial function in CKD.

The endothelium is sensitive to mechanical stimuli such as shear stress that occurs with increases in blood flow. Elevated shear stress has been shown to augment L-arginine uptake in cultured endothelial cells in a dose-dependent manner [115] while exercise training has been shown to augment L-arginine uptake in the forearm vasculature of heart failure patients [107]. Additionally, exercise training is well known for its ability to improve nitric oxide synthesis though activation and increased protein expression of the endothelial nitric oxide synthase (eNOS) resulting in improved endothelial function [8, 62, 142, 159]. The known cardiovascular benefits of aerobic exercise combined with its potential to improve the L-arginine transport system make exercise training an attractive therapy to reduce cardiovascular risk in CKD. Voluntary wheel running has previously been shown to prevent the development of vascular dysfunction in 5/6 nephrectomized rats when initiated immediately after surgery [133]; however, little is known about the benefits of aerobic exercise on vascular function once CKD has already been established. Likewise, the effect of exercise on the L-arginine transport system in CKD has not been tested. The purpose of this study was to determine if four weeks of voluntary wheel running could improve L-arginine transport and vascular function in CKD. We hypothesized that

voluntary wheel running would result in an increase in CAT-1 protein expression that would ultimately lead to an improvement in the utilization of dietary L-arginine and vascular function.

3.2 Methods

3.2.1 Ethical Approval

All procedures and experiments were approved by the University of Delaware Institutional Animal Care and Use Committee and were conducted in accordance with the Public Health Service Policy regarding the Humane Care and Use of Laboratory Animals. Animals were individually housed and kept on a 12-hour light/dark cycle and were fed a standard rat chow diet with free access to food and water.

3.2.2 Animal Model and Study Groups

12-week old male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) were used for this study. All rats were placed in a standard rat cage that was equipped with a running wheel for an initial period of one week to allow for familiarization with the wheels. After this initial period, rats were randomly assigned to one of two groups and underwent either 5/6 ablation/infarction surgery to induce CKD, or a similar sham surgery to serve as a control. Animals were anesthetized using isoflurane (1.5-5.0%) and kept on a heating pad to control body temperature during surgery. Surgery was performed using sterile technique and consisted of ligating 2/3 of the blood supply to the left kidney, followed by complete removal of the right kidney. The sham animals had both kidneys exposed and manipulated without any ablation or infarction. After 4 weeks of disease development, CKD animals either remained sedentary (SED) or received one of the following

interventions: supplemental L-arginine monohydrochloride (ARG; 1.25g/L in drinking water; Sigma Aldrich #A5131), voluntary wheel running (RUN), or combined RUN+ARG. The dose of L-arginine was chosen based on previous studies in which 1.25 g/L L-arginine administered in the drinking water prevented the development of renal and endothelial dysfunction respectively [6, 164].

3.2.3 Assessment of Renal Function

Animals were kept for 8 weeks after surgery and were monitored for the development of kidney disease. This time frame is sufficient for animals to develop moderate to severe kidney disease [27], endothelial dysfunction, glomerulosclerosis, and decreased cardiac function [86, 90] confirming the development of both CKD and cardiovascular disease in this model. Renal function was assessed in all groups by measuring urine protein excretion, serum creatinine, and blood urea nitrogen (BUN). Urine samples were obtained from an overnight collection (16 hours) in a metabolic cage at baseline, 4 and 8-weeks post surgery. During this time, animals were allowed access to water *ad libitum;* however, they were restricted from food consumption to prevent contamination of urine samples. Urine volumes were recorded and samples were aliquoted and stored at -80°C until later assay of protein concentration using the Bradford method [19]. Protein excretion was calculated as the product of urine flow (mL/hour) and protein concentration (mg/mL) and expressed as mg protein/24hr/100g body mass.

Blood samples were obtained from the vena cava immediately prior to sacrifice. Samples were stored at 4°C for 10 minutes, centrifuged at 3,000 RPM for 10 minutes at 4°C and serum was obtained and stored at -80°C for later assay of serum creatinine and blood urea nitrogen (BUN). Prior to analysis, serum samples were

filtered with 10kDa Amicon-Ultra centrifugal filters (EMD Millipore Corporation; Billerica, MA) to remove debris and the filtrate was used for analysis. Serum creatinine was determined based on a Jaffe reaction between creatinine and picrate in which the reaction product was measured at an absorbance of 510 nm. BUN was determined from the conversion of urea to L-glutamate by urease and glutamate dehydrogenase where a change in absorbance at 340 nm is directly related to BUN concentration.

3.2.4 Preparation of Tissue

Animals were anesthetized with an intraperitoneal injection of ketamine/xylazine (100mg/kg) and subsequently sacrificed by exsanguination via removal of the heart. The thoracic aorta was quickly dissected and placed in ice-cold physiological salt solution (PSS; NaCl, 118.99 mmol; KCl, 4.69 mmol; $CaCl_2$-$2H_2O$, 2.50 mmol; $MgSO_4$-$7H_2O$, 1.17 mmol; KH_2PO_4, 1.18 mmol; EDTA, 0.03 mmol; Glucose, 1.091 g/L; $NaHCO_3$ 2.100 g/L; pH, 7.4). Aortas were cleaned of any fat and connective tissue and cut into 3mm ring sections for assessment of vascular function, or L-arginine transport as described below. The remaining aortic tissue was snap-frozen in liquid nitrogen and stored at -80°C for later determination of protein expression. To determine if wheel running resulted in improved skeletal muscle oxidative capacity, the right soleus muscle was carefully dissected, snap frozen in liquid nitrogen, and stored at -80°C for later analysis of citrate synthase activity (Sigma Aldrich, St. Louis, MO).

3.2.5 Vascular Function Studies

Vascular function was assessed *in vitro* using isometric ring experiments in aortic ring segments. Rings from each animal were mounted onto wire force transducers within individual organ chambers (DMT 610M, Danish Myotechnology). Rings were oxygenated with carbogen gas (5% CO_2, 95% O_2) and kept under physiological conditions at 37°C and pH 7.4 in normal PSS. Vessels were stretched to a resting tension of 20mN and allowed to equilibrate over the course of an hour. Following equilibration, rings were constricted with a single dose of phenylephrine (3×10^{-7} M) and relaxed with a single dose of acetylcholine to test the viability of the endothelium.

Once viability was confirmed, rings were again constricted with a single dose of phenylephrine (PE; $3x10^{-7}$M) and dose-dependently relaxed with cumulative doses of acetylcholine (Ach; 10^{-9}-10^{-5} M). Upon completion of the first dose-response experiment, rings were washed with PSS every 10 minutes for 1 hour and then treated with either vehicle (PSS) or 10μM of the NOS inhibitor L-NAME (Sigma-Aldrich) to determine if impaired endothelium-dependent relaxation was NO-mediated. Rings were again constricted and dose-dependently relaxed to Ach as previously described above. Finally, endothelium-independent relaxation to the NO donor, sodium nitroprusside (SNP; 10^{-9}-10^{-5} M) was assessed dose-dependently in a subset of rings.

3.2.6 L-arginine Transport

L-arginine transport was assessed in aortic rings using an established *in vitro* technique [72, 90, 128, 129]. Aortic rings were prepared into 3mm long ring sections, cut lengthwise into strips, and placed in HEPES buffer at 37°C. Ring segments were then incubated for 60 seconds in HEPES buffer containing 1mM L-arginine and 10μCi

L-[^{3}H]arginine. A separate set of rings were incubated in buffer containing 1mM L-arginine but no L-[^{3}H]arginine to serve as a control. Rings were then washed with ice-cold PBS and homogenized in RIPA buffer. Tissue homogenates were analyzed for radioactivity (disintegrations per minute) by liquid-scintillation counting using a Packard Tri-Carb 2900-TR liquid scintillation counter and protein content was determined using the BCA protein assay (Thermo Scientific).

3.2.7 Western Blot

Protein expression of CAT-1, PKCα, eNOS, and phosphorylated eNOS (Ser 1177) were determined by Western Blot. Aortic tissue samples were homogenized at a 1:20 dilution in lysis buffer containing (150mM NaCl, 1.0% Triton X-100, 0.5% SDS, and 50mM Tris HCl pH 8.0) with added protease inhibitor (Sigma Aldrich, #P8340) and phosphatase inhibitor cocktails (Sigma Aldrich, #P0044) using glass on glass homogenization. Homogenates were centrifuged at 10,000g for 10 min at 4^{o}C and supernatant was extracted and prepared for analysis. Protein concentration was determined using the Bradford assay [19] and samples were diluted in sample buffer containing β-mercaptoethanol and boiled for 5 minutes. Samples were loaded into either 12% (CAT-1; PKCα) or 8% (eNOS; eNOS-P1177) Tris-HCl gels and electrophoresed for 60 minutes at 100V. Gels were transferred to a nitrocellulose membrane, blocked, and immunoblotted with the primary antibody for the protein of interest: CAT-1 (1:200; Santa Cruz sc-66825), PKCα(1:100; Santa Cruz sc-8393), eNOS (1:1000; BD 610297), eNOS-P1177(1:500; BD 612393). Membranes were washed and incubated with the appropriate recommended secondary antibody, treated with ECL developing reagent (Thermo Scientific) and developed on film. Data are

presented as intensity relative to β-actin (1:2000; Santa Cruz Biotechnology sc-81178). Western blots were quantified using Image J software from NIH.

3.2.8 Statistical Analysis

All data were assessed for normality and homoscedasticity. Data that were non-normally distributed were log transformed and analysis was performed on transformed data. Urinary protein excretion was analyzed using a 2-way repeated-measures ANOVA. All other data were analyzed using a one-way ANOVA and a Tukey's post hoc test was performed to assess differences between groups. Dose response curves were generated for all vascular function data using GraphPad Prism 5.0 software and normalized to % relaxation. Dose response curves were fit with a non-linear regression line and LogEC50, maximal relaxation or constriction (Emax), and area under the curve (AUC) were determined. The alpha level was set at 0.05 and all data are presented as means ± SEM.

3.3 Results

Table 3.1 contains body mass and measurements of renal function. Body mass was significantly reduced in SED and ARG-treated animals compared to SHAM and was restored by RUN and RUN+ARG interventions (Table 3.1). Urinary protein excretion was unaltered in SHAM animals and was progressively elevated in all of the CKD groups at 4 and 8 weeks post-surgery. Serum creatinine and BUN were significantly elevated above SHAM animals to similar levels at 8 weeks in all CKD groups. No differences in renal function were observed among any of the CKD groups at any time point indicating no effect of treatment on renal function. Animals in the RUN and RUN+ARG group ran a similar distance over the course of the 4-

week intervention (Table 3.2). Citrate synthase activity was attenuated in SED and RUN+ARG animals compared to SHAM and decreased in ARG and RUN animals, but did not reach statistical significance. Animals in the ARG and RUN+ARG group consumed a similar amount of L-arginine in their drinking water (Table 3.2).

Table 3.1 Body Mass and Renal Function Measurements

	SHAM	**SED**	**ARG**	**RUN**	**RUN+ARG**
Body Mass (g)	432 ± 9	376 ± 13 *	376 ± 18 *	402 ± 9	388 ± 17
Urine Protein Excretion ($mg \cdot 24\ hr^{-1} \cdot 100g^{-1} BM$)					
Baseline	6.2 ± 0.7	5.3 ± 0.8	5.8 ± 0.5	6.2 ± 0.7	7.2 ± 1.5
Week 4	4.5 ± 0.8	42.4 ± 5.8 **	50.3 ± 12.2 **	35.7 ± 4.5 **	41.4 ± 7.0 **
Week 8	6.0 ± 0.8	51.7 ± 6.1 **	48.4 ± 9.4 **	53.5 ± 6.7 **	65.5 ± 7.1 **
Serum Creatinine (mg/dL)	1.3 ± 0.1	3.9 ± 0.4 **	4.6 ± 0.5 **	4.6 ± 0.6 **	4.4 ± 0.5 **
BUN (mg/dL)	25.5 ± 0.9	72.15 ± 2.5 *	71.5 ± 2.1 *	69.3 ± 2.6 *	68.3 ± 4.5 *

Values are mean ± SEM. * $p < 0.05$ vs SHAM. ** $p < 0.001$ vs SHAM. n = 8 in each group

Table 3.2 Intervention Characteristics

	SHAM	SED	ARG	RUN	RUN+ARG
Total Running Distance (m)	---	---	---	8882 ± 1487	9538 ± 1750
Soleus Citrate Synthase Activity (μmol/ml/min)	590.6 ± 28.7	455.6 ± 17.5 *	497.9 ± 32.5	524.0 ± 18.6	495.2 ± 18.0 *
Total L-Arginine Consumption (mg)	---	---	2040 ± 152	---	2193 ± 135

Values are mean ± SEM. * $p < 0.05$ vs SHAM. n = 8 in each group

3.3.1 Vascular Function Studies

The role of nitric oxide in mediating the endothelium-dependent relaxation response to acetylcholine was confirmed in all animals by an abolished relaxation response to the NOS inhibitor L-NAME (data not shown). The complete EDR dose-response to acetylcholine in aortic rings is presented in Figure 3.1A. Endothelial function was significantly impaired in both SED and ARG animals as indicated by a reduction in the area under the curve (AUC) of the dose-response curve (Figure 3.1B). Running improved AUC above SED animals in both RUN and RUN+ARG treated animals. RUN+ARG had the greatest effect overall and was significantly improved above ARG animals (Figure 3.1B). Maximal effect of Ach (Emax) was significantly attenuated in both SED and ARG-treated animals. Emax was not significantly different from SHAM animals and was improved above SED in the RUN+ARG group only (Figure 3.1C). Endothelium-independent relaxation to SNP was not impaired in SED animals compared to SHAM and was not improved by any of the treatments. A small but significant reduction in logEC50 to SNP was observed in RUN+ARG animals (Table 3.3).

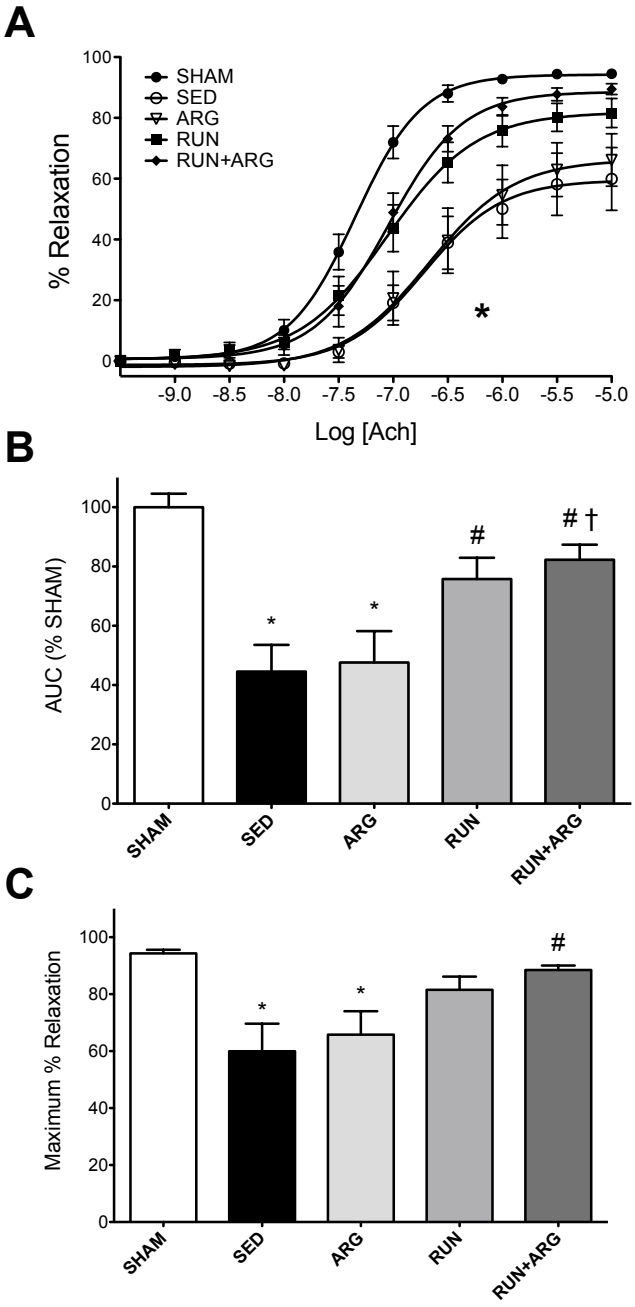

Figure 3.1: **Endothelium-Dependent Relaxation (EDR).** (A) Dose-response (logEC50) (B) area under the curve, and (C) maximal response of aortic rings to acetylcholine. $p < 0.05$: * vs. SHAM; # vs. SED; † vs. ARG. n = 8 in each group.

Table 3.3 Endothelium Independent Response to SNP

	n	AUC	Emax	Log EC50
SHAM	7	100 ± 3.68	100.57 ± 0.89	-8.56 ± 0.13
SED	8	87.65 ± 4.75	97.36 ± 1.11	-8.17 ± 0.15
ARG	7	84.45 ± 7.26	99.11 ± 1.21	-8.03 ± 0.22
RUN	8	85.15 ± 2.30	98.02 ± 0.72	-8.08 ± 0.11
RUN+ARG	7	80.83 ± 4.02	96.56 ± 1.20	-7.77 ± 0.15 *

Values are mean ± SEM. n, number of animals; * $p < 0.05$ vs SHAM

3.3.2 Tissue Analysis

L-arginine transport was attenuated in both SED and ARG-treated animals as indicated by a reduction in aortic uptake of L[^{3}H]-arginine compared to SHAM controls. L-arginine transport was improved above SED animals in the RUN group. The greatest improvement occurred in the RUN+ARG animals in which L-arginine transport was greater than both SED and ARG animals (Figure 3.2B). Protein expression of the L-arginine transporter CAT-1 was attenuated in SED animals compared to SHAM and was unaltered by any of the treatments (Figure 3.2A), despite observing an overall improvement in L-arginine transport. PKCα has previously been shown to inhibit L-arginine transport in CKD. We observed a significant increase in PKCα protein expression in our SED animals only, whereas both RUN and RUN+ARG animals returned to similar levels as SHAM (Figure 3.3). Protein expression of eNOS was not significantly different among groups; however, phosphorylation of eNOS at ser-1177 was attenuated in sedentary CKD animals compared to sham and was not improved by any of the treatments.

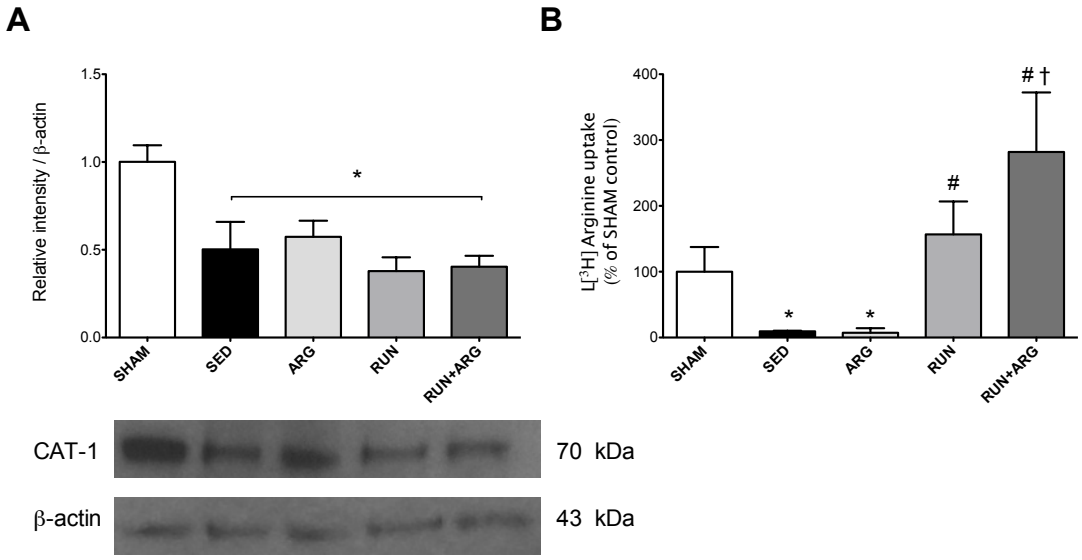

Figure 3.2: **Aortic CAT-1 Protein Expression and L-arginine Uptake.** (A) Aortic CAT-1 protein expression normalized to β-actin assessed by western blot; n = 8 in each group. (B) L-[^{3}H] arginine uptake, disintegrations per minute (DPM) normalized to protein content; n = 5 in each group. $p < 0.05$: * vs. SHAM; # vs. SED; † vs. ARG.

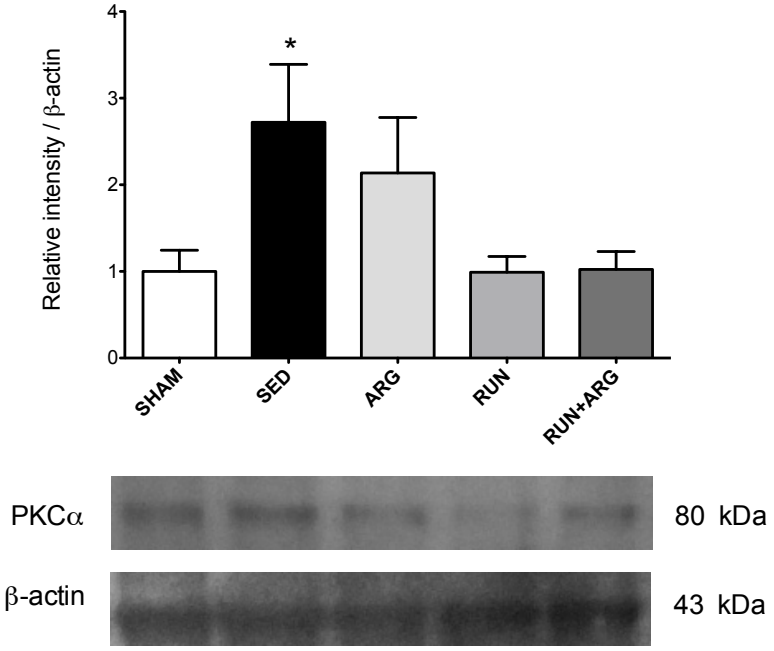

Figure 3.3: **PKCα Protein Expression.** Normalized to β-actin assessed by western blot. * $p < 0.05$ vs. SHAM. n = 8 in each group.

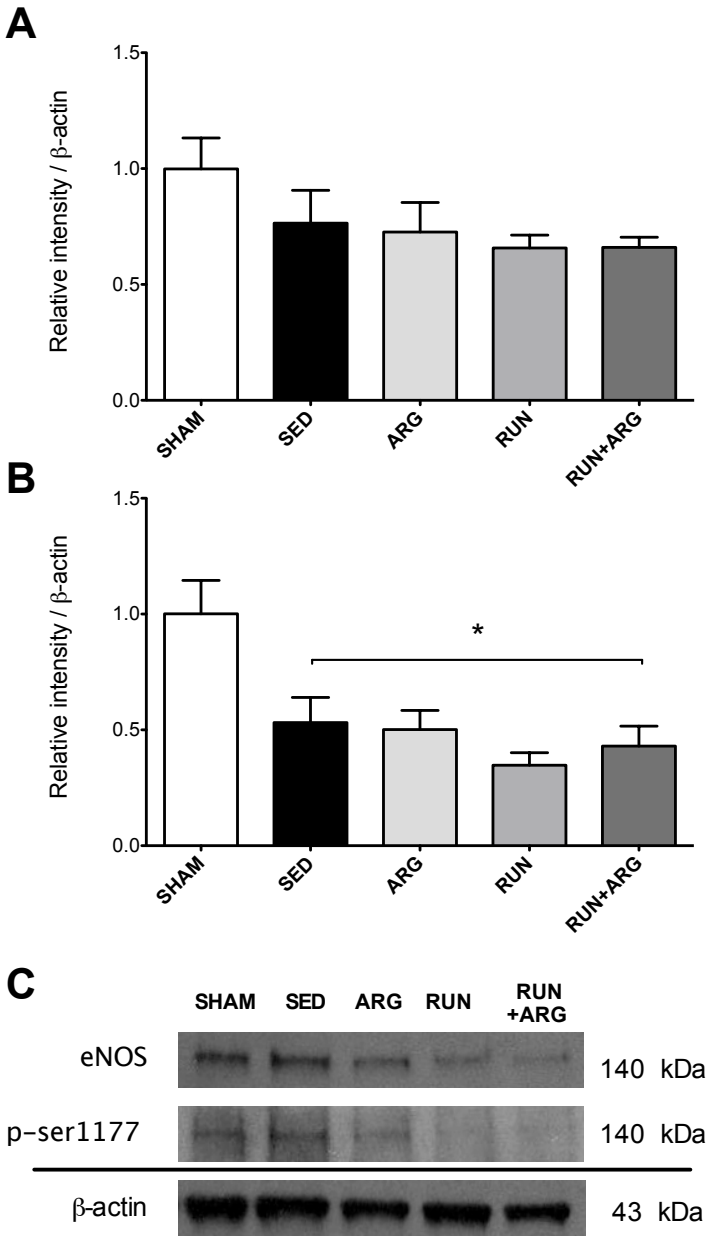

Figure 3.4: **eNOS Protein Expression and Phosphorylation.** (A) Total eNOS and (B) phosphorylated eNOS (ser-1177) in aorta normalized to β-actin assessed by western blot with (C) representative blots. n = 8 in each group.* $p < 0.05$ vs. SHAM.

3.4 Discussion

The primary finding of this study is that 4 weeks of voluntary wheel running reversed vascular dysfunction in rats with CKD. This effect was greatest in animals that received L-arginine in combination with the wheel running intervention but was not seen in animals that received L-arginine alone. The improvement in vascular function was not associated with increased CAT-1 expression but was accompanied by augmented L-arginine transport and a reduction in PKCα protein expression. The results of this study suggest that exercise may be a beneficial therapy to reverse vascular dysfunction in CKD, in part by improving in the vascular uptake of L-arginine.

The beneficial effect of exogenous L-arginine on vascular function has been well documented across a wide range of pathologies including hypercholesterolemia, diabetes mellitus, coronary artery disease, and congestive heart failure [15, 31, 33, 51, 110, 147] as well as in primary aging [15, 24, 66]. Although endothelial cells maintain a sufficient intracellular concentration of L-arginine to satisfy the Km of eNOS, treatment with exogenous L-arginine has been shown to cause improvements in endothelial NO synthesis. This has been referred to as the "L-arginine Paradox" and suggests that NO is derived from extracellular sources of L-arginine [94, 135]. Classified as a semi essential amino acid, the body is normally capable of producing sufficient quantities of L-arginine to sustain homeostasis [119]; however, the synthesis of L-arginine occurs primarily in the kidneys and is impaired in CKD [10, 27]. Supplementation with exogenous L-arginine would therefore be highly beneficial to patients with CKD. To that end, L-arginine has been shown to prevent the progression of renal [6, 120] and endothelial [164] dysfunction in rats when treatment was initiated immediately following renal mass reduction. Though important, these findings do not

provide insight into the role of L-arginine on vascular function once CKD has already been established. Unfortunately clinical studies in patients with CKD have not been as successful [11, 29, 36, 40]. In the present study, we initiated L-arginine treatment 4 weeks after surgery in order to ensure that renal and vascular impairments had already developed. Using this model, we were unable to improve vascular dysfunction in our ARG-treated animals suggesting that L-arginine is not effective at reversing endothelial dysfunction once CKD has already been established. Our findings are in agreement with clinical studies that demonstrate no improvement in endothelium-dependent dilation response to either an acute infusion of L-arginine in adult pre-dialysis patients [36], nor to oral L-arginine supplementation in children with chronic renal failure [11].

The lack of improvement with L-arginine is not entirely surprising and may explain a unique mechanism by which endothelial function is impaired in CKD. The transport of extracellular L-arginine into the endothelium occurs primarily through the cationic amino acid transporter CAT-1 via a sodium and pH independent mechanism [32]. L-arginine transport has been shown to be impaired in endothelial cells cultured in uremic plasma [163] as well as in animal models of CKD [72, 90, 128], and is accompanied by a decline in CAT-1 protein expression in the vasculature. We confirmed these findings in the present paper as demonstrated by a significant reduction in CAT-1 protein expression and L[^{3}H]-arginine uptake in SED and ARG-treated animals. In this context, impaired vascular transport of L-arginine explains the lack of improvement in endothelial function observed in our ARG treatment group.

CAT-1 forms a caveolar complex with the NO-producing enzyme, eNOS [87, 94] suggesting a mechanism by which extracellular L-arginine is delivered directly to

eNOS at the endothelial cell surface. NO synthesis is improved by endothelial shear stress such as that which occurs with increased blood flow during exercise. This adaptation occurs at the onset of exercise via an increase in eNOS phosphorylation at ser-1179 [8] as well as in response to long term exercise training through increased eNOS expression [131]. Interestingly, we did not detect a significant attenuation in eNOS protein expression; however, phosphorylation at ser-1177 was decreased in all groups with CKD and was unaltered by voluntary wheel running. The duration and intensity of the wheel running intervention may not have been great enough to cause changes in eNOS expression or activation. Improvements in vascular function with exercise however, are not limited to changes in eNOS expression and activity as porcine aortic endothelial cells exposed to 40 minutes of shear stress exhibited improved L-arginine uptake [115]. Likewise, 8 weeks of aerobic and light resistance exercise training augmented forearm vascular uptake of L-arginine in humans with congestive heart failure [107]. Our results confirm the beneficial effects of exercise on vascular function as animals that received access to a running wheel (RUN) improved their vascular function by ~30% above SED animals. The greatest effect however, was observed in animals that received the running wheel in combination with L-arginine (RUN+ARG) as noted by an increase in vascular function by nearly 40% above both SED and ARG-treated animals. These results suggest that physical activity is not only capable of augmenting endothelial function in CKD, but that it does so in part by improving the utilization of L-arginine.

Our initial hypothesis was that exercise would improve vascular function by increasing protein expression of the L-arginine transporter, CAT-1. Interestingly, the improved relaxation observed in RUN and RUN+ARG animals did not appear to

occur through an increase in CAT-1 protein expression; however, wheel running did improve CAT-1 transport activity as evidenced by an increase in L[^{3}H]-arginine uptake. This suggests that exercise mediates an increase in L-arginine uptake through a post-translational modification of CAT-1. Previous work by Ingbir et al., (2008) reported similar results in which treatment of uremic rats with rosiglitazone improved L-arginine uptake in aortic rings without increasing CAT-1 protein expression. Rosiglitazone treatment was associated with a decrease in aortic PKCα protein expression and an increase in CAT-1 phosphorylation suggesting that PKCα is an important mediator of CAT-1 transport activity [72]. In the present study, we observed an increase in PKCα expression in the aortas of SED animals whereas levels were returned to similar levels as SHAM animals in both RUN and RUN+ARG treatment groups.

The exact mechanism by which PKCα mediates a reduction in L-arginine transport is not entirely clear. Activation of PKCα with phorbol 12-myristate 13-acetate (PMA) has been shown to result in internalization of CAT-1 to the cytosol in *X. laevis* oocytes and was shown to occur independent of CAT-1 phosphorylation [121]. In another study conducted in pulmonary artery endothelial cells, PMA activation of PKCα reduced L-arginine uptake without changing the expression or subcellular distribution of CAT-1 [83]. Whether PKCα mediates a decrease in L-arginine transport through a direct reduction in CAT-1 catalytic activity or by inducing its translocation to the cytosol in CKD is not completely understood. In our experiments, the magnitude of increase in L[^{3}H]-arginine uptake with wheel-running exceeded that of SHAM control animals suggesting exercise may influence L-arginine

transport at least in part through a direct alteration in CAT-1 catalytic activity; however, future work is needed to elucidate this mechanism.

Our findings are particularly interesting given the low volume of physical activity recorded in both running groups. Adams et al. (2005) reported running distances of ~ 7km/day in both sham rats and rats that underwent 5/6 nephrectomy when animals were given access to a running wheel during the initial stages of disease development [1]. In our study, RUN and RUN+ARG animals only ran ~9km over the course of the entire 4 week intervention period. The fact that we observed such robust improvements in L-arginine uptake and endothelial function with such a low volume of exercise is surprising and may provide clinical relevance and rational for future translational studies. Patients with CKD exhibit low tolerance to aerobic exercise [74]; however, our results suggest that even small increases daily physical activity could have profound effects on vascular function.

In conclusion, 4 weeks of voluntary wheel running improved vascular function in rats with CKD through a mechanism related to improved L-arginine transport. This mechanism was not related to an increase in CAT-1 protein expression but was associated with a reduction in PKCα protein expression, suggesting post-translational modification of CAT-1. Treatment with exogenous L-arginine has not been successful in patients with CKD and our data suggest that exercise may play an important role in the regulation of the L-arginine-nitric oxide axis. Future work should consider the L-arginine transport mechanism as a therapeutic target to treat cardiovascular disease in humans with CKD and should further explore the role of increased physical activity in improving vascular health.

Chapter 4

CONCLUSION

4.1 Summary

The overall goal of this project was to explore the mechanisms of endothelial dysfunction in an animal model of chronic kidney disease and to test the hypothesis that endothelial function could be improved by increased physical activity. The heightened risk of cardiovascular disease, combined with a sedentary lifestyle in patients with CKD, provide clinical relevance to our results. Furthermore, our findings that physical activity improved the utilization of L-arginine in animals with CKD provides a platform for future translational research in human patients and adds to our understanding of how "the arginine paradox" is influenced by kidney disease.

In our first series of experiments, we explored mechanisms of impaired endothelial function that are thought to contribute to reduced L-arginine availability. Our results suggested that increased competition from arginase is not a likely mechanism in CKD as we did not detect a difference in arginase protein expression or enzymatic activity and were unable to improve endothelial function with arginase inhibition. This conclusion was consistent with that of Chen et al. (2012) who found no increase in aortic arginase expression in rats treated with a high dose of puromycin aminonucleoside (PAN) to induce severe CKD [26]. Arginase activity is inversely correlated to urea concentration and was suppressed in liver homogenates from rats 6 weeks following 5/6 nephrectomy as well as in experimental conditions of uremia [100]. These findings are in support of our own and provide insight into why arginase

is not a likely contributor to vascular dysfunction in CKD. Whether arginase contributes to the pathogenesis of endothelial dysfunction earlier in the development of CKD is less clear. Low-dose administration of PAN to induce less severe CKD was associated with increased arginase expression [26]. Future studies should explore the role of arginase inhibition at earlier stages of CKD when the uremic burden is less severe.

While arginase did not seem to contribute to vascular dysfunction in our model of moderate to severe CKD, our results did point us towards the involvement of the L-arginine transport system in endothelial dysfunction. In contrast to the majority of the literature in other disease models, we were unable to restore EDR with an exogenous dose of L-arginine. This occurred in the absence of superoxide as acute treatment with Tempol alone or in combination with L-arginine did not improve relaxation. This finding was coupled with a decline in both L[^{3}H]-arginine transport and protein expression of the primary L-arginine transporter, CAT-1 and is in accordance with studies by Schwartz et al. (2006) in which L-arginine transport was impaired in nephrectomized rats [128]. Taken together, the results of these studies suggested that arginase is not a contributor to endothelial dysfunction in CKD; however, impaired L-arginine transport may play an important role in diminishing substrate availability for NO production leading to endothelial dysfunction.

Our second set of experiments was designed to target the L-arginine transport system using a voluntary wheel running intervention. Evidence from cell culture experiments supports the role of shear stress in inducing L-arginine transport [115], and exercise training has been shown to be effective at improving L-arginine uptake in humans with chronic heart failure [107]. We tested the effect of 4 weeks of voluntary

wheel running and/or L-arginine supplementation on vascular function in rats with CKD. Our initial hypothesis was that L-arginine supplementation by itself would be ineffective due to impaired vascular uptake. We further hypothesized that voluntary wheel running would improve vascular function in part by increasing L-arginine uptake such that L-arginine supplementation would become effective only when treated in combination with wheel running. The majority of interventional studies involving L-arginine or exercise in animal models of CKD have focused on treating vascular dysfunction immediately following surgery. Our approach was novel in that it allowed for significant disease progression prior to initiating our interventions. This insured that any effect observed was likely due to reversal and not simply a delay in the progression of vascular dysfunction.

As predicted, animals that received L-arginine supplementation alone did not differ from their sedentary counterparts; however, improvements were seen in animals that underwent voluntary wheel running. The biggest improvements however, were observed in animals that received L-arginine in combination with wheel running, suggesting that exercise increased the ability to utilize L-arginine. These results were supported by the finding that L[^{3}H]-arginine uptake was augmented in animals that underwent voluntary wheel running. While the results supported our overall hypothesis, they seemed to do so through a different mechanism than we originally anticipated. Contrary to our initial hypothesis, wheel running did not improve CAT-1 protein expression suggesting instead that the observed increase in L[^{3}H]-arginine uptake was related to a post-translational modification of CAT-1.

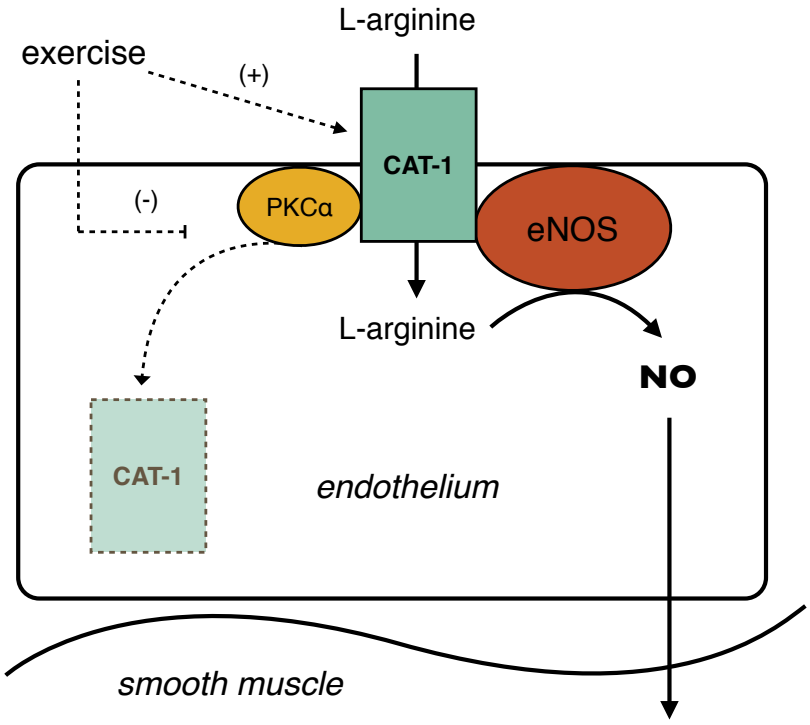

Figure 4.1: **Working Model of Exercise and Vascular Function in CKD.** Impairments in L-arginine uptake are mediated by activation of PKCα resulting in internalization of CAT-1 to the cytosol [121]. Exercise improves L-arginine transporter in part by decreasing PKCα expression resulting in maintenance of CAT-1 at the endothelial surface. Exercise may also directly stimulate L-arginine transport though a presently unknown mechanism.

Regulation of L-arginine transport activity has been linked to activation of PKCα resulting in translocation of CAT-1 into the cytosol [121] where it is subsequently ubiquitinated and tagged for degradation by the lysosome [156]. Ingbir et al. (2008) has shown that treatment of uremic rats with rosiglitazone attenuated the increase in PKCα and improved CAT-1 transport activity independent of changes in CAT-1 protein expression [72]. Our results are similar in that we found a decrease in PKCα in the aortas of animals that received wheel running, accompanied by an increase in L-arginine transport. Interestingly, the magnitude of increase in L-arginine transport was not only restored, but exceeded that of sham animals. Taken together, these findings suggest that exercise improves L-arginine transport in CKD by limiting the PKCα internalization of CAT-1, but may also be involved in the direct activation of CAT-1 (Figure 4.1); however, the specific mechanisms by which this might occur are presently unknown.

4.2 Perspectives

The ability of exogenous L-arginine to augment nitric oxide production, despite a seemingly sufficient intracellular concentration, has long been a topic of interest in vascular physiology. That this phenomenon appears to be absent in patients and animals with CKD provides a unique window into the role of the "L-arginine Paradox" in CKD. The present studies not only demonstrate the potential benefit of increased physical activity on vascular function in CKD, but also indicate an important role of physical activity in reversing impairments in L-arginine transport. Our studies suggest that while L-arginine itself may not be an effective therapy to treat vascular dysfunction in CKD, combining L-arginine treatment with light to moderate physical activity may be beneficial.

Our findings are particularly interesting in that they suggest that positive vascular benefits can be obtained from a very low volume of physical activity. CKD patients typically have poor tolerance to exercise accompanied by a sedentary lifestyle. The ability to achieve robust improvements in cardiovascular function with only minor increases in daily levels of physical activity could have a profound effect on the management of patients with CKD. Future work should explore the relationship between moderate levels of physical activity and vascular function in humans with CKD. Overall, our findings in a preclinical model of CKD have the potential to lead to clinically relevant studies regarding the use of exercise as an adjunct therapy to reduce the burden of cardiovascular disease in patients with CKD.

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Appendix A

IACUC APPROVAL

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University of Delaware
Institutional Animal Care and Use Committee
Application to Use Animals in Research and Teaching

(Please complete below using Arial, size 12 Font.)

Title of Protocol: Endothelial Progenitor Cells and Cardiovascular Function in Chronic Kidney Disease	
AUP Number: 1200-2009-0	**← (4 digits only — if new, leave blank)**
Principal Investigator: David Edwards	
Common Name: Rat **Genus Species:** Rattus norvegicus Sprague Dawley	
Category Assigned: *(please mark one)* ☐ A. None to slight or momentary pain or distress ☐ B. Pain or distress will be alleviated by drugs or other means ■ C. Pain or distress will not be alleviated	

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IACUC Approval Signature: _____

Date of Approval: 3/18/09

University of Delaware
Institutional Animal Care and Use Committee
Application to Use Animals in Research and Teaching

(Please complete below using Arial, size 12 Font.)

Title of Protocol: Non pharmacological interventions in chronic kidney disease	
AUP Number: 1227-2012-0	← (4 digits only — if new, leave blank)
Principal Investigator: David G. Edwards	
Common Name: Rat **Genus Species:** Rattus norvegicus Sprague Dawley	

Pain Category: ***(please mark one)***

USDA PAIN CATEGORY: *(Note change of categories from previous form)*	
Category	**Description**
☐ **B**	Breeding or holding where NO research is conducted
☐ **C**	Procedure involving momentary or no pain or distress
■ **D**	Procedure where pain or distress is alleviated by appropriate means (analgesics, tranquilizers, euthanasia etc.)
☐ **E**	Procedure where pain or distress cannot be alleviated, as this would adversely affect the procedures, results or interpretation

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IACUC Approval Signature: Patricia L. Buttrey DVM

Date of Approval: 12/1/2011

Appendix B

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