THE EFFECT OF SHEAR STRESS AND UREA ON ENDOTHELIAL CAT-1 EXPRESSION

by

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LIST OF ABBREVIATIONS

ADMA, asymmetric dimethylarginine

A/I, ablation/infarction

ANOVA, analysis of variance

BH_4, tetrahydrobiopterin

CaM, calmodulin

CAT-1, cationic amino acid transporter-1

CKD, chronic kidney disease

CVD, cardiovascular disease

EDR, endothelium-dependent relaxation

eNOS, endothelial nitric oxide synthase

FMD, flow-mediated dilation

GFR, glomerular filtration rate

NO, nitric oxide

NOS, nitric oxide synthase

O_2^-, superoxide

$ONOO^-$, peroxynitrite

PAEC, pulmonary artery endothelial cells

PAN, puromycin aminonucleoside

PKCα, protein kinase C alpha

PTX, pertussis toxin

sGC, soluble guanylyl cyclase

SOD, superoxide dismutase

ROS, reactive oxygen species

UT, urea transporters

ABSTRACT

Chronic Kidney Disease (CKD) affects over 26 million people in the United States and has an estimated prevalence of 11.5%. CKD is associated with an elevated risk of cardiovascular disease (CVD), even if traditional cardiovascular risk factors are not present. This may be due in part to a reduction in the bioavailability of the vasodilator nitric oxide (NO) and an impairment in the transport of the NO substrate L-arginine into the endothelium. Cell culture studies have shown that uremic toxins such as urea play a role in the inhibition of L-arginine transport into endothelial cells, ultimately leading to endothelial dysfunction. L-arginine is transported into the endothelium through the cationic amino acid transporter CAT-1, which is regulated by PKCα. Evidence from previous studies suggests that PKCα phosphorylates CAT-1, directly altering and decreasing its catalytic activity, or that PKCα causes internalization of CAT-1 to the inside of the cell where it can no longer participate in L-arginine transport. Increased endothelial shear stress, which occurs during physical activity as a result of increased blood flow, has been shown to improve L-arginine uptake into endothelial cells. Interestingly, previous studies have shown that in the 5/6 ablation/infarction (A/I) rat model of CKD, 4 weeks of voluntary wheel running improved L-arginine uptake, however, this did not occur through an increase in CAT-1 expression. However, in sedentary CKD animals, PKCα expression was increased compared groups that participated in voluntary wheel running. Additionally, in uremic rats, treatment with rosiglitazone, a PPARγ agonist and anti-diabetic drug that

has been shown to be beneficial in CKD, was associated with a decrease in PKCα, as well as a decrease in phosphorylated CAT-1 compared to untreated rats. The benefits of physical activity to potentially decrease the expression of PKCα thereby improving L-arginine uptake through a decrease in phosphorylated CAT-1 makes exercise a potential treatment to reduce the risk of cardiovascular disease in CKD. Our overall hypothesis was that shear stress alters CAT-1 expression and the expression of its phosphorylated state.

In our first set of experiments, we studied the effect of different shear stress levels on CAT-1, PKCα, and phosphorylated CAT-1 expression. As shear stress increased, CAT-1 expression also increased, with expression at 20 dyn/cm^2 being significantly greater than 0 dyn/cm^2 ($p < 0.05$). PKCα and phosphorylated CAT-1 expression decreased as shear levels increased starting at 10 dyn/cm^2.

In the second set of experiments, we explored the effect of different shear stress levels on CAT-1, PKCα, and phosphorylated CAT-1 expression in the presence of 25mM of the uremic toxin, urea. CAT-1 expression remained unchanged with all shear stress levels, suggesting that shear stress may result in a post translational effect of CAT-1. Both PKCα and phosphorylated CAT-1 expression were significantly decreased at all shear levels compared to 0 dyn/cm^2 ($p < 0.05$). This decrease in phosphorylation of CAT-1 by PKCα could in turn lead to enhanced L-arginine transport and vascular function. These results suggest that activities that increase shear stress, such as exercise, may be a beneficial therapy in improving vascular function in patients suffering from CKD.

Chapter 1

INTRODUCTION

1.1 Chronic Kidney Disease and Cardiovascular Disease

Chronic Kidney Disease (CKD) is a condition that results in renal damage and decreased kidney function over time. CKD has become a growing health problem and has an estimated prevalence of 11.5% (Ricardo et al. 2015). Patients suffering from CKD can be placed into one of five stages based on the glomerular filtration rate (GFR) as well as persistent albuminuria (Table 1.1; Figure 1.1) (Coresh et al. 2007).

CKD has been shown to be very strongly associated with Cardiovascular Disease (CVD). Cardiovascular risk factors, impaired kidney function, and increased albumin in the urine increase the risk of CVD by two to four times, and heart failure is also doubled in those with early CKD (Gansevoort et al. 2013). CVD significantly increases the risk of morbidity and mortality in patients with CKD by as much as ten to thirty times (Rahman et al. 2014, Sarnak et al. 2003). In people with kidney failure, sudden cardiac death accounts for 26% of deaths compared to just 6-13% for the general population (Gansevoort et al. 2013). It is also important to note that patients with CKD are actually more likely to die from CVD than progress to end stage renal disease. Furthermore, the risk of developing CVD in CKD is present even in patients who lack traditional CVD risk factors such as old age, high HDL cholesterol, and diabetes mellitus (Sarnak et al. 2003).

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

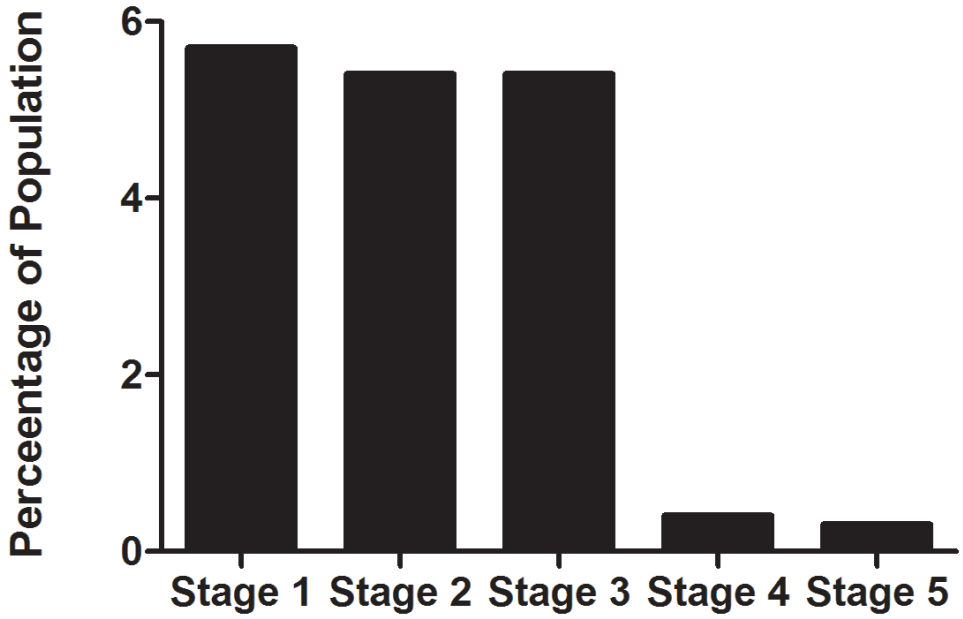

Figure 1.1 Percentage of population aged ≥ 20 years in each stage of CKD. Adapted from Sarnak et al 2003 and the Centers for Disease Control and Prevention website (www.cdc.gov).

1.2 The Vascular Endothelium

It has been well established that those with CKD have a high risk of developing CVD compared to the general population due in part to endothelial dysfunction. The inner lining of blood vessels is made up of a single layer of endothelial cells, known collectively as the endothelium. Although these cells are only a simple monolayer acting as a barrier between the interstitial space and blood vessels, they have many other important functions involving the regulation of vascular homeostasis (Rajendran et al. 2013). These functions include the control of inflammatory responses through assisting or inhibiting the formation and degradation of blood clots, aiding in smooth muscle growth and proliferation, and the modulation of vascular tone through the synthesis and release of vasoactive substances (Deanfield et al. 2007, Cahill and Redmond 2016). Vascular tone, which is altered in CKD, is a critical component of vascular health referring to the state of contraction or relaxation of a blood vessel. As stated previously, the endothelium maintains vascular tone through the release of several vasoactive substances such as nitric oxide (NO), endothelium-derived hyperpolarizing factor, and prostacyclin, as well as endothelium-derived contracting factors including endothelin-1 and thromboxane. One of the main substances the endothelium is responsible for synthesizing and releasing is the endothelium derived relaxing factor NO. In addition to maintaining vascular tone, NO has several other roles such as the inhibition of platelet adherence and aggregation, regulating endothelial permeability, inhibiting smooth muscle proliferation (Davignon and Ganz 2004). As can be seen in Figure 1.2, an increase in blood flow-induced shear stress to the vessel leads to an increase in NO production. This is carried out

through the uptake of the amino acid L-arginine into the endothelium primarily through the cationic amino acid transporter, CAT-1. Endothelial nitric oxide synthase (eNOS) then converts L-arginine into NO, which diffuses into the smooth muscle and activates the enzyme soluble guanylyl cyclase (sGC). sGC catalyzes the conversion of GTP into cyclic GMP (cGMP), which further results in vascular relaxation (Walther et al. 2004). Therefore, NO is vital in maintaining vascular health, as mechanisms that disrupt its synthesis and release have been shown to contribute to endothelial dysfunction and atherosclerosis (Sitia 2010).

1.2.1 L-arginine Transport Via CAT-1

NO is produced from the amino acid L-arginine, so its synthesis depends on the transport of L-arginine into the endothelium. L-arginine uptake into the endothelium occurs primarily through the cationic amino acid transporter, CAT-1. CAT-1 consists of 14 transmembrane domains and transports cationic L-amino acids in both a Na^+ and pH independent manner. CAT-1 exhibits K_M values for L-arginine, L-lysine, and L-ornithine of 100-150 μM with a strong preference for transport into cells (Closs 2006).

It has been reported that CAT-1 is expressed in all mammalian tissues except for the liver (Ito and Groudine 2007). It is not uniformly distributed over the cell surface, but rather is concentrated in randomly distributed clusters within the plasma membrane. In endothelial cells, CAT-1 is seen in close proximity to eNOS due to the fact that they are co-localized within caveolae, which may provide a mechanism of extracellular L-arginine to eNOS for NO synthesis (McDonald et al. 1997). This NO synthesis depends upon an adequate and continual supply of L-arginine. Several

studies have reported that the intracellular L-arginine concentrations are well above what is necessary for eNOS to be completely saturated (Hecker et al. 1990). Therefore, increasing the extracellular L-arginine should not increase NO production any further, but studies indicate that NO production by endothelial cells can be increased by extracellular L-arginine, despite a saturating intracellular concentration (McDonald et al. 1997). In fact the intracellular concentration of L-arginine in endothelial cells can be over 100-fold without changing NO production. This is what is known as the "arginine paradox." This observation could be explained by the existence of a caveolar complex between CAT-1 and eNOS, resulting in the directed delivery of extracellular L-arginine to eNOS for NO synthesis (McDonald et al. 1997).

1.2.2 Endothelial Nitric Oxide Synthase (eNOS)

Nitric oxide synthases (NOS) are a family of enzymes that produce NO. There are three NOS isoforms: neuronal NOS, inducible NOS, and endothelial NOS (eNOS). eNOS is present in endothelial cells and is therefore responsible for the synthesis of endothelium derived NO (Andrew and Mayer 1999).

The functional form of eNOS is a homodimer that uses several co-factors in order to stabilize its structure, such as calmodulin (CaM), 5,6,7,8-tetrahydrobiopterin (BH_4), zinc, flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN). Regulation of eNOS occurs variously through the availability of these cofactors, post-translational modifications, protein-protein interactions, and cellular localization, which can all lead to changes in overall NO production (Shu 2015).

Differential posttranslational modifications of eNOS could alter eNOS function and activity. For example, different vascular beds regulate eNOS activity through phosphorylation (Pan 2009). Also, shear stress within blood vessels impacts

the activity of eNOS. Large arteries tend to be sensitive to shear stress, while areas with low shear stress experience lower NO bioavailability and increased plaque development. One study reported that in rabbit carotid arteries in areas of high shear stress, eNOS is upregulated and activated through the phosphorylation of serine 1177 (Boo et al. 2002, Chiu and Chien 2011).

eNOS activity is also regulated by the binding of proteins and cellular localization. Interactions of eNOS with the scaffolding protein caveolin-1 anchors the enzyme on the plasma membrane where it can interact with additional cellular components needed for NO synthesis (Andrew et al. 1999). Other proteins also interact with eNOS and activate NO production. These proteins include heat shock protein 90 (HSP90), NOS interacting protein (NOSIP), ß-actin, and calmodulin. These binding partners cause eNOS translocation from the plasma membrane and ultimately NO synthesis (Su 2014).

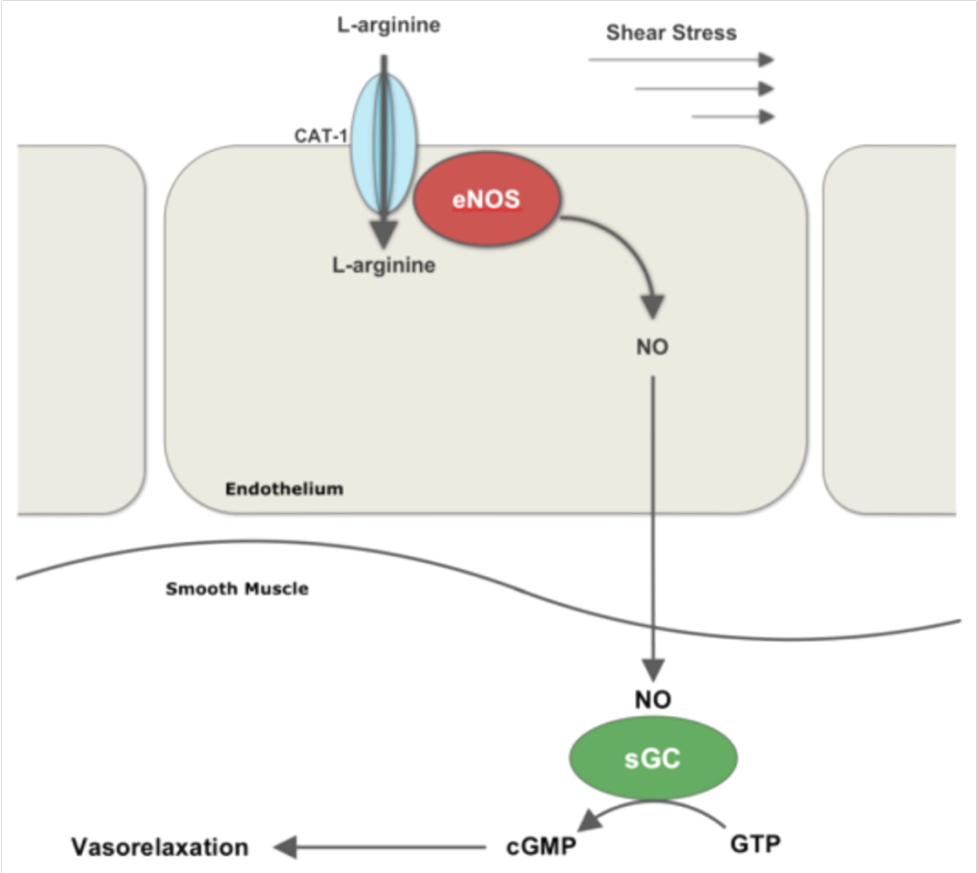

Figure 1.2 **Pathway of L-arginine Transport.** L-arginine is transported into the endothelium through the transporter CAT-1 and is converted into NO by eNOS. NO diffuses into smooth muscle where it acts on sGC to convert GTP into cGMP, leading to vasorelaxation.

1.3 Endothelial Dysfunction in CKD

Endothelial dysfunction and ultimately cardiovascular disease mortality in those with CKD is a result of insufficient availability of nitric oxide. Mechanisms that affect the release of NO, such as reduced L-arginine synthesis and transport into the endothelium, as well as oxidative stress, play a role in this endothelial dysfunction that is present in CKD (Sitia et al. 2010), as can be seen in Figure 1.3. Impairments in endothelium dependent relaxation observed in renal failure patients have been assessed via measurements of forearm blood flow by Annuk et al., (2000). These impairments were eliminated in the presence of the endothelium-independent vasodilator sodium nitroprusside, which indicates dysfunction of the endothelium and not the smooth muscle function, ultimately leading to the development of atherosclerosis and cardiovascular disease (Annuk et al. 2000). Additionally, a study conducted by Cross et al., (2003) demonstrated a reduction in forearm blood flow in pre-dialysis patients suffering from end stage renal disease. When patients were administered the antioxidant vitamin C, endothelium dependent dilation was increased (Cross et al. 2003), which suggests that oxidative stress plays a role in endothelial dysfunction (Cross et al. 2003 and Nanayakkara et al. 2007). However, when the patients were administered vitamin C together with the NOS inhibitor L-NMMA, or endothelium independent dilators, there was no increase in forearm blood flow. These observations suggest that the reduction in forearm blood flow is NO-dependent rather than an impairment in smooth muscle function (Cross et al. 2003). Similarly, vascular function can also be improved in patients with mild to moderate renal impairment

through the use of antioxidants. Flow mediated dilation was improved in these patients following an antioxidant intervention including pravastatin, vitamin E, and homocysteine-lowering therapy. Improvements in vascular function occurred despite continuous decline of renal function (Nanayakkara et al. 2007). Furthermore, activation of NADPH oxidases by angiotensin II has been suggested as a possible contributor to oxidative stress and endothelial dysfunction in CKD. Short term ACE-inhibition in humans with stage 1 CKD resulted in improvements in endothelium dependent relaxation (EDR) as assessed by flow mediated dilation (FMD) (Yilmaz et al. 2009).

The formation of the endogenous NOS inhibitor, asymmetric dimethylarginine (ADMA), may also contribute to endothelial dysfunction in CKD (Chen et al. 2012). ACE inhibition may reduce ADMA levels, leading to an improvement in endothelial function. Yilmaz et al., (2007) showed that CKD patients treated with the ACE inhibitor Ramipril or the angiotensin II receptor antagonist Valsartan reduced ADMA concentrations and increased FMD (Yilmaz et al. 2007).

1.3.1 Impaired L-arginine Transport

L-arginine supplements have been shown to increase NO production and therefore enhance EDR in both humans and animals suffering from a variety of conditions (Tiradentes et al. 2015, Clarkson et al. 1996, Clemmensen et al. 2013, Creager et al. 1992), indicating that impairments in L-arginine uptake and availability for NO synthesis play a role in endothelial dysfunction in CKD.

L-arginine is synthesized from the precursor L-citrulline by the enzymes argininosuccinate synthase (ASS) and argininosuccinate lysate (ASL). In a 5/6 ablation/infarction (A/I) rat model of CKD, reductions in abundance of both of these

enzymes were observed. Also, there was an elevation in plasma citrulline concentration in these rats, which would suggest that there was a decrease in the amount of L-arginine being converted from L-citrulline (Chen and Baylis 2010).

In addition to an impairment in substrate synthesis, reduced L-arginine transport into the endothelium and in turn reduced substrate availability for NO synthesis has been demonstrated in patients with CKD (Guldener et al. 1998). Despite there being an intracellular concentration of L-arginine exceeding what is necessary to saturate eNOS (Hardy and May 2002), exogenous L-arginine has still been shown to increase NO production. This suggests that NO production relies on extracellular sources of the amino acid (McDonald et al. 1997). This also implies that there is a decrease in the amount of L-arginine available in those with disease conditions that can be reversed through the use of exogenous L-arginine.

As stated previously, exogenous L-arginine increases NO production (McDonald et al. 1997). From this, one might assume that the use of exogenous L-arginine would be beneficial in treating impaired renal function; however, this is not necessarily the case. In 5/6 nephrectomized rats given an oral supplement of L-arginine for 9 weeks, the development of CKD by reduced NO synthesis was prevented (Ashab et al. 1995, Yamamizu et al. 2007). On the other hand, when an L-arginine infusion was given to patients in predialysis renal failure, it was not effective in increasing EDR (Cross et al. 2001). The findings of these studies suggest that even though L-arginine may prevent the development of endothelial dysfunction in early stages of CKD, it is not as effective once CKD is already present.

Uremic toxins also play a role in the attenuation of L-arginine transport through CAT-1 and reduced substrate availability for NO production (Xiao et al.

2001). The presence of uremic toxins may provide an explanation for why exogenous L-arginine is ineffective in treating late stage CKD. Endothelial cells cultured in uremic plasma and cells cultured in synthetic solutions of uremic levels of urea experience impairments in L-arginine transport (Xiao et al. 2001). Impaired L-arginine transport has also been shown in rat models of CKD that was also associated with a decrease in CAT-1 expression and activity (Martens et al. 2014 and Schwartz et al. 2006). It is important to note that the urea must be transported through endothelial urea transporters (UT) into the cells in order to cause this effect; when the UT inhibitor phloretin was added to the solutions, it prevented the decline in L-arginine transport into cells treated with high levels of urea (Wagner et al. 2002). In addition to urea, the endogenous L-arginine derivative, asymmetric dimethylarginine (ADMA) is increased in CKD and leads to NO deficiency through competitive inhibition of eNOS. ADMA is made during protein methylation by protein arginine methyltransferase (PRMT)1 and is released following proteolysis (Chen et al. 2012). The majority of ADMA is removed by enzymatic degradation by dimethylarginine-dimethylaminohydrolase (DDAH) (Assar et al. 2016). In those suffering from CKD, PRMT expression and activation and ADMA production is increased (Vallance et al. 1992), while DDAH activity is attenuated (Chen et al. 2012), so ADMA clearance by the kidney is impaired, leading to an overall increase in the plasma concentration of ADMA. Even though increased plasma concentrations of ADMA have been observed in CKD, ADMA does not seem to contribute to attenuations in L-arginine transport into the endothelium. In cultured endothelial cells, uremic levels of ADMA (10μM) did not inhibit L-arginine transport; transport was only inhibited by supraphysiological concentrations of ADMA (0.1mM-2mM) (Xiao et al. 2001). This suggests that these

increases in ADMA result in endothelial dysfunction through competitive inhibition of eNOS and therefore decreased NO synthesis, rather than affecting L-arginine transport (Vallance et al. 1992). This competitive inhibition could also provide a possible explanation for the "arginine paradox," the observation that exogenous L-arginine in vitro or in vivo increases NO production despite baseline concentrations of L-arginine that should saturate eNOS (Caplin and Leiper 2012).

L-arginine availability for NO synthesis may also be affected by competition from the enzyme arginase (Chung et al. 2014). Arginase regulates NO bioavailability by competing with eNOS for their common substrate L-arginine. Arginase catalyzes the conversion of L-arginine to L-ornithine and urea and can result in reduced NO production by shunting L-arginine away from the eNOS pathway to the arginase pathway (Shemyakin et al. 2012). Inhibition of arginase has been confirmed to restore endothelial function in a variety of conditions, such as obesity (Chung et al. 2014), hypertension (Zhang et al. 2004), atherosclerosis (Hwang et al. 2015), and aging (Berkowitz et al. 2003) through improved NO production. Inhibition of arginase has also been shown to delay the progression of renal failure. In rats that underwent 5/6 A/I surgery, inhibition of arginase with a manganese (Mn^{2+}) free diet suppressed arginase activity in the liver and kidney, delaying the progression of renal failure due to improved NO production (Sabbitini et al. 2003). Even though arginase may be significant in the early development of endothelial dysfunction, it does not seem that arginase contributes to endothelial dysfunction in late-stage CKD. Arginase inhibition was not able to restore EDR in aortic rings of rats 8 weeks after 5/6 A/I induced CKD. Additionally, aortic arginase expression and activity were not affected in these animals, suggesting that arginase does not play a role in endothelial dysfunction in

moderate to severe CKD (Martens et al. 2014). Similarly, in rats treated with a high dose of puromycin aminonucleoside (PAN) to induce severe CKD, there was no increase in aortic arginase expression; however, rats treated with a low dose of PAN as a model of moderate CKD showed a significant increase in arginase expression (Chen et al 2012). The role of arginase on endothelial function in severe CKD may be explained by suppression via a negative feedback mechanism. Arginase is responsible for converting L-arginine into urea, which is a uremic toxin retained in the blood of those suffering from CKD. 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 late stage CKD (Martens et al. 2014). Moradi et al., (2006) has demonstrated this type of uremic inhibition in liver homogenates from rats following 5/6 nephrectomy (Moradi et al. 2006). This mechanism may offer an explanation for the findings of Chen et al., (2012) who saw no change in arginase in rats treated with a high dose of PAN (Chen et al. 2012). These studies suggest that arginase does not contribute to late-stage CKD and provides a possible explanation as to why arginase inhibition does not restore endothelial function in more severe stages of CKD (Martens et al. 2014).

In addition to reduced substrate availability and delivery for NO synthesis, oxidative stress contributes to endothelial dysfunction in CKD primarily through NOS dependent pathways. NO is a free radical, making it a susceptible target of redox reactions (Guzik et al. 2006). NADPH oxidase is responsible for generating the superoxide anion (O_2^-), which has a high affinity for reacting with NO to produce peroxynitrite ($ONOO^-$), limiting the amount of NO available for vasodilation (Sung et al. 2013). Administration of antioxidants has been shown to improve endothelial

function in CKD. In 5/6 nephrectomized rats, vitamin E increased NO production, decreased NO inactivation, and improved endothelial function (Vaziri et al. 2002). Antioxidant therapy can also be beneficial in humans with mild to moderate CKD; Nanayakkara et al., (2007) found that a treatment strategy including vitamin E resulted in a significant improvement in endothelial function and urinary albumin excretion (Nanayakkara et al. 2007). Superoxide dismutase (SOD) is the antioxidant responsible for sequestration of O_2^- to H_2O_2, and the administration of Tempol, a SOD-mimetic drug, has also shown to have beneficial effects on endothelial dysfunction and CKD (Ding et al. 2015). In 5/6 nephrectomized rats, the administration of Tempol resulted in improved endothelial function, reduced blood pressure, and mitigated the progression of renal disease in CKD (Ding et al. 2015 and Hasdan et al. 2002).

Additionally, the uncoupling of eNOS can lead to reduced NO bioavailability. Oxidation of the cofactor BH_4 results in unstable eNOS that becomes functionally uncoupled, reducing NO production and enhancing the generation of O_2^- (Moens et al. 2011). Supplementation with BH_4 has been shown to be effective in restoring endothelial dysfunction. Yamimizu et al., (2007) demonstrated that in 5/6 nephrectomized rats, supplementation with BH_4 restored EDR of aortic rings (Yamimizu et al. 2007). In addition, Podjarny et al., (2004) found that 5/6 nephrectomized rats administered BH_4 had decreased systolic blood pressure and proteinuria (Podjarny et al. 2004).

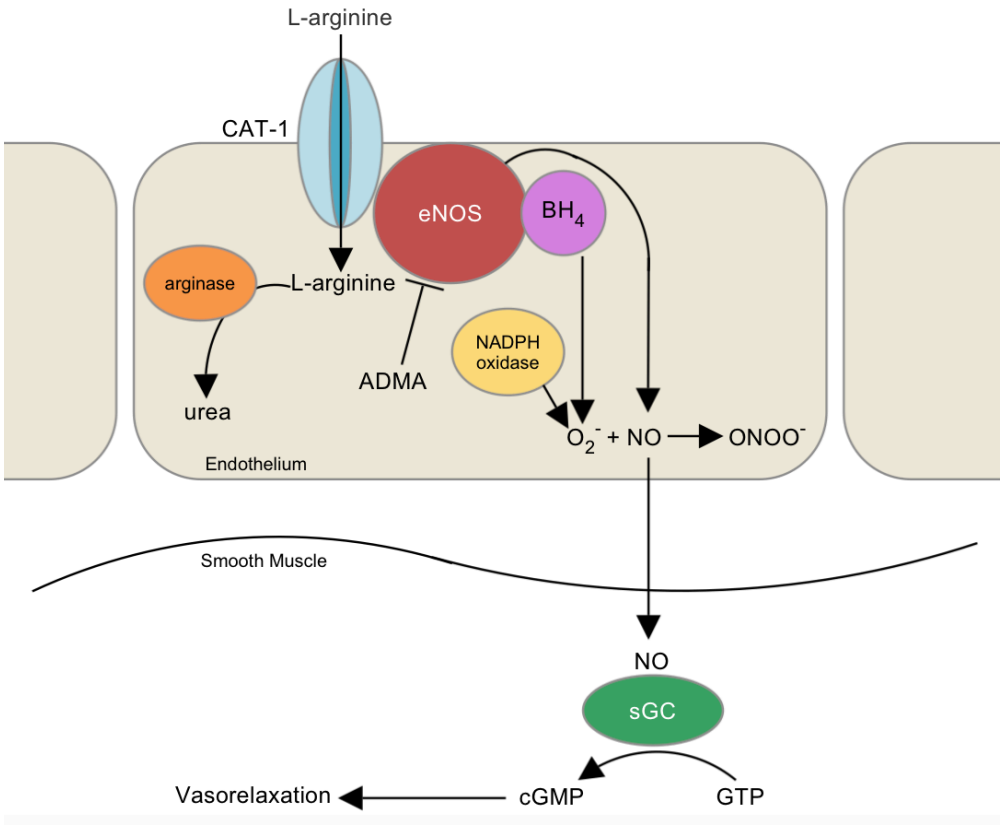

Figure 1.3 Mechanisms of Endothelial Dysfunction in CKD. L-arginine is transported through CAT-1 into the endothelium where it is converted to NO by eNOS. NO diffuses into smooth muscle where it acts on sGC to catalyze the conversion of GTP into cGMP, resulting in vasorelaxation. Reduced availability of L-arginine due to competition from arginase or competitive inhibition of eNOS by ADMA can lead to endothelial dysfunction. Also, oxidative stress from increased O_2^- synthesis can result in reduced NO availability through the combination of O_2^- with NO forming $ONOO^-$. Additionally, oxidation of the cofactor BH_4 can lead to uncoupling of eNOS and further O_2^- synthesis.

1.3.2 PKCα and Regulation of CAT-1

L-arginine transport occurs primarily through the transporter CAT-1. CAT-1 contains three putative sites for phosphorylation by protein kinase C alpha (PKCα) localized in the fifth and sixth intracellular loops (Rotmann et al. 2004). Additionally, both CAT-1 and activated PKCα have been shown to be localized in caveolae, which allows for the possibility of CAT-1 and PKCα interaction (Mineo et al. 1998). Taken together, these findings provide evidence that PKCα is involved in regulating CAT-1 activity. More specifically, this regulation of CAT-1 by activated PKCα leads to reduced L-arginine transport activity. Ingbir et al., (2008) demonstrated this association in rats with CKD and treatment with rosiglitazone. In these CKD rats, there was a decrease in both CAT-1 expression and L-arginine uptake. When treated with rosiglitazone, L-arginine uptake returned to normal, but CAT-1 expression remained in a reduced state, which could signify a posttranslational effect on CAT-1 (Ingbir et al. 2008). In order to investigate this, they also looked at PKCα and phosphorylated CAT-1 expression levels. PKCα levels were increased in the CKD rats, and this effect was prevented by rosiglitazone, which is an anti-diabetic drug and PPARγ agonist. Also, phosphorylated CAT-1 was increased in these animals as well, and rosiglitazone abolished this increase in phosphorylation (Ingbir et al. 2008). Several other studies have also examined the effect of PPARγ agonists on vascular function. For example, (Scoditti et al. 2009) found that in endothelial cells, rosiglitazone reduced VEGF and PMA-stimulated PKCα translocation to the membrane. This results in decreased VEGF-induced COX-2 expression, a pro-angiogenic enzyme, ultimately leading to the inhibition of angiogenesis (Scoditti et al

2009). Additionally, PPARγ agonists have also been shown to increase the release of nitric oxide from endothelial cells (Calnek et al. 2003), as well as inhibiting the development of atherosclerosis in LDL receptor-deficient mice (Li et al. 2000).

The observed decrease in L-arginine transport seems to be due to the internalization of CAT-1 into the cytosol (Rotmann et al. 2004). In *X. laevis* oocytes, internalization of CAT-1 occurred as a result of the PKCα activating phorbol ester, PMA. This internalization of CAT-1 was accompanied by a similar reduction in L-arginine uptake. This would indicate that the transport inhibition from PKCα is the result of CAT-1 internalization as opposed to modifications in CAT-1 catalytic activity (Rotmann et al. 2004).

Krotova et al., (2003) also found that in pulmonary artery endothelial cells (PAECs), PMA induces a change in CAT-1, most likely through a posttranslational modification. However, it was not as a result of a change in location of the transporter as Rotmann et al., (2004) reported. When PMA activates PKCα, this activation is known to result in targeting of plasma membrane caveolae, where CAT-1 is localized. This suggests that the translocation of PKCα to membrane caveolae induces phosphorylation of CAT-1, leading to inhibition of L-arginine transport (Krotova et al. 2003). Depletion of PKCα was shown to promote dephosphorylation of CAT-1 and activation of transport activity. In this case, it is likely that PKCα directly regulates CAT-1 transport activity rather than regulating through downstream signaling pathways that may involve ERK1/2 or p38 MAP kinase; inhibitors of MAP kinase and p38 kinase were not found to change the effects of PMA on L-arginine uptake in PAECs (Krotova et al. 2003).

Similarly, Zharikov et al., (2007) also demonstrated that in PAECs, transduction cascades are not involved in the effects of treatment with pertussis toxin (PTX), an activator of L-arginine transport. In endothelial cells, PTX can change the activity of several kinases, such as p42/p44 MAPK, PKC, and p38 MAPK, which are all targeted to caveolae where CAT-1 is localized as well. This creates the possibility that PTX activates L-arginine transport in endothelial cells through one of these signaling cascades. However, when PAECs were treated with inhibitors of the Rho kinase, MAP kinase, and phosphatidylinostirol 3 kinase pathways, L-arginine transport was not affected and the PTX-induced activation of L-arginine transport remained unchanged (Zharikov et al. 2007).

1.4 Exercise and Vascular Function

Exercise training has been shown to be very beneficial in improving endothelial dysfunction in several disease states, such as aging and heart failure, and could potentially have similar effects in CKD. Even in the presence of cardiovascular risk factors such as hypertension, diabetes, smoking, and obesity, regular physical activity improves cardiovascular function (Walther et al. 2004). Parnell et al., (2005) demonstrated that 8 weeks of exercise training, including walking, cycling, and hand weights, in patients with heart failure is very effective in the augmentation of endothelial function through an increase in L-arginine transport (Parnell et al. 2005). Exercise training has also been shown to improve endothelial dependent dilation (Green et al. 2004, Griffin et al. 1999, Thompson et al. 2004) through the upregulation of eNOS (Hambrecht et al. 2000, Zhou et al. 2010) and increased synthesis of NO (Nyberg et al. 2012, Sun et al. 1994). Additionally, exercise training increases vascular antioxidant capacity, decreases oxidative stress, and improves NO bioactivity

(Rush et al. 2003). Donato et al., (2010) found that giving an antioxidant cocktail to elderly men put through exercise training improved vascular function through an antioxidant-induced reduction in circulating free radicals (Donato et al. 2010).

1.4.1 Exercise and L-arginine Transport

L-arginine supplementation may help improve exercise capacity and alter reactive oxygen species (ROS) metabolism, suggesting a close association between L-arginine and exercise in respect to vascular function (Rush et al. 2003). In hypercholesterolemic mice, administration of L-arginine over 4-8 weeks normalized exercised induced endothelium dependent NO synthesis through exercise hyperemia, and also prevented a decline in aerobic exercise capacity that is normally seen in these mice. This effect in combination with observations of reduced limb blood flow, exercise capacity, and post exercise nitrogen oxide in hypercholesterolemic animals indicated that both exercise hyperemia and exercise capacity depends on the NOS pathway (Maxwell et al. 2001). Similarly, Lomonosova et al., (2014) showed that L-arginine supplementation prior to eccentric exercise preserved exercise performance capacity in rats, in addition to also preventing muscle fiber damage (Lomonosova et al. 2014). Exercise in combination with L-arginine supplementation may improve endothelial dysfunction that can result from an insufficient availability of L-arginine, and this combination may be additive with regard to correcting endothelial dysfunction (Hambrecht et al. 2000). In chronic heart failure patients, supplemental oral L-arginine improved endothelium-dependent relaxation, and the effects of this supplementation with exercise training on EDR seemed to be additive. These findings suggest that improved endothelial function may be an additive effect of increased substrate availability and upregulation of eNOS (Hambrecht et al. 2000). This can

also be seen in rats with CKD (Martens et al. 2014). 4 weeks of voluntary wheel running reversed vascular dysfunction through an increase in L-arginine uptake. This effect was greatest in the groups that received oral L-arginine supplementation in combination with the wheel running. However, this effect was not observed in animals that received the L-arginine alone. This demonstrates that even small amounts in physical activity may be beneficial to correcting vascular dysfunction and suggest that exercise may work partly by improving the uptake of L-arginine (Martens et al. 2014).

In addition to this, L-arginine has also been shown to have a protective role against oxidative stress (Bailey et al. 2010). In exercise trained rats, levels of SOD activity were significantly increased, and when given L-arginine supplementation, these elevations were reversed, indicating a protective effect of L-arginine resulting from a decreased generation of O_2^-. Also, exercised rats with L-arginine treatment maintained their body weight, while rats without treatment experienced a significant loss in body weight, indicating that L-arginine reduces oxidative stress and enhances the antioxidation capabilities in exercise trained rats (Shan et al. 2013). Furthermore, L-arginine supplementation and exercise has been shown to improve blood flow and cardiovascular function relating to decreased oxidative stress in type 1 diabetic patients (Fayh et al. 2013), as well as upregulating the antioxidant defense system after myocardial infarction in rats (Ranjbar et al. 2016).

1.4.2 Exercise and Role of PKCα in L-arginine Transport

PKCα also plays a part in CAT-1 transport activity and therefore L-arginine transport by extension. In 5/6 nephrectomized rats, EDR was significantly decreased, however, this was reversed when the animals were given L-arginine supplementation

and participated in voluntary wheel running (Martens et al. 2014). Interestingly, this observed improvement in EDR did not occur through an increase in CAT-1 expression. However, the voluntary wheel running did in fact improve CAT-1 transport activity, as there was an increase in L-arginine uptake. This finding suggests that exercise mediates an increase in L-arginine uptake through a posttranslational modification of CAT-1 (Martens et al. 2014). PKCα is known to be an important mediator of CAT-1 transport activity. Uremic rats treated with rosiglitazone had increased levels of L-arginine uptake that was not accompanied by an increase in CAT-1 expression. The treatment of rosiglitazone was associated with a decrease in PKCα expression and an decrease in CAT-1 phosphorylation (Ingbir et al. 2008). In rats with CKD that remained sedentary for 4 weeks, there was an increase in PKCα expression, while groups with wheel running and L-arginine supplementation had normal levels. This increase in PKCα expression suggests that PKCα may be acting on CAT-1 and phosphorylating it, leading to an attenuation of L-arginine uptake (Martens et al. 2014). However, this mechanism by which PKCα mediates a reduction in L-arginine transport is not fully understood; PKCα may cause a direct reduction in CAT-1 catalytic activity, or it may induce the translocation of CAT-1 to the cytosol. (Rotmann et al. 2004) found that the activation of PKCα with PMA resulted in internalization of CAT-1 to the cytosol of *Xenopus laevis* oocytes independently of CAT-1 phosphorylation. However, in another study conducted, Krotova et al., (2003) found that PMA activation of PKCα reduced L-arginine uptake in pulmonary artery endothelial cells without changing the expression or distribution of CAT-1. Whether PKCα directly affects the activity of CAT-1 or changes its location remains unclear.

1.5 Shear Stress

Exercise training is accompanied by an increase in blood flow, resulting in increased endothelial shear stress. Shear stress, a biomechanical force generated by flowing blood and tissue flow (Obi et al. 2014), is sensed by the endothelium through mechanotransduction, which is mediated by several mechanosensing molecules including junctional proteins, receptor kinases, focal adhesions, G-protein-coupled receptors, ion carriers, and glycocalyx. Shear stress influences glycocalyx, which are carbohydrate-rich proteoglycans anchored to the plasma membrane of endothelial cells (Chistiakov et al. 2016). Blood flow deflects chains of proteoglycan molecules, which then deform the apical plasma membrane, leading to force transmission (Gulino-Debrac 2013). The glycocalyx is especially important because it has a vasoprotective role in preventing leukocyte and platelet adhesion and mediating shear stress dependent endothelial NO release (Chistiakov et al 2016), which is why shear stress is beneficial to vascular function. Studies using the proatherogenic Apo lipoprotein-E null mice demonstrate that chronic reduction of carotid artery shear stress impairs endothelial function and promotes atherosclerotic lesions, confirming the notion that shear stress is critical for sustaining optimal vascular health (Cheng et al. 2006). Also, it has been shown that remaining sedentary for prolonged periods of time results in endothelial dysfunction mediated by a reduction in shear stress, further emphasizing this importance of shear stress and therefore exercise (Restaino et al. 2016). Similarly, increases in shear levels result in improvements in endothelial function. Greyling et al., (2015) showed that elevations in shear stress were able to prevent hyperglycemia-induced declines in FMD; in fact, a significant increase in FMD was observed (Greyling et al. 2015). These findings highlight the significance of shear stress and exercise with regard to vascular function.

1.5.1 Shear Stress and L-arginine

Shear stress is a potent stimulus for increased L-arginine uptake and NO synthesis, which would explain why increased blood flow by way of exercise is beneficial to vascular function (Weber et al. 2005). Shear stress may improve NO production via increases in substrate delivery to the endothelium. Posch et al., (1999) showed that increases in shear stress resulted in increases in L-arginine transport in a stress-dependent manner in porcine aortic endothelial cells. This increase in L-arginine transport is involved in NO synthesis and impairments in this pathway could lead to endothelial dysfunction (Weber et al. 2005). When cells are incubated in glycated LDL from patients with diabetes, shear stress-induced L-arginine uptake was abolished (Posch et al. 1999). These findings suggest increased shear stress may be effective in treating endothelial dysfunction that occurs as a result of impaired L-arginine transport.

1.5.2 Shear Stress and NO Production

Shear Stress as a result of increased blow flow leads to in an increase of eNOS expression and activity, thereby activating NO synthesis. This NO synthesis can occur in both a calcium-dependent and calcium-independent manner. eNOS is classified as a Ca^{2+}/Calmodulin (CaM)-dependent enzyme, and so it can be activated by various agonists after an increase in the intracellular concentration of free Ca^{2+}(Dimmeler et al. 1999). For example, HDAC5 is known to have a role in shear stress-mediated eNOS expression. Shear stress can stimulate HDAC5 phosphorylation through a Ca^{2+}/Calmodulin-dependent pathway, leading to the dissociation of HDAC5 and MEF2 and enhanced MEF2 transcriptional activity. This then results in the stimulation of KLF-2 expression, which regulates many flow-responsive genes such as

eNOS, thereby increasing its expression (Wang et al. 2010). On the other hand, shear stress-induced phosphorylation of eNOS at the serine 1177 residue by Akt activation enhances eNOS enzyme activity independently of an increase in Ca^{2+} (Dimmeler et al. 1999). This shear stress-induced activation of Akt and eNOS is modulated by the tyrosine phosphorylation of platelet endothelial cell adhesion molecule-1 (PECAM-1), as evidenced by an abolishment of phosphorylation of these molecules when treated with the tyrosine kinase inhibitor PP1 (Fleming et al. 2005).

In addition to acute increases in NO synthesis in response shear stress, long-term exposure to shear stress can also result in improvements in NO production. Human retinal microvascular endothelial cells exposed to 24 and 48 hours of shear stress displayed increased expression of eNOS mRNA expression (Ishibazawa et al. 2011). In addition to an increase in eNOS transcription, shear stress also results in the posttranscriptional regulation of eNOS via stabilization of eNOS mRNA. Endothelial cells exposed to shear stress showed an increase in expression of eNOS transcripts associated with 3' polyadenylation. These transcripts were more stable than those of nonsheared cells, and eNOS mRNA from sheared cells was found to be more actively translated (Weber et al. 2005). Furthermore, shear stress has been associated with increased levels of eNOS activity. With increasing levels of shear stress, eNOS activity increases in a stress-dependent manner. This shear stress-mediated NO formation is also dependent upon extracellular arginine (Posch et al. 1999). Extracellular L-arginine drives NO production even when intracellular levels of L-arginine are available in excess, a phenomenon known as the "arginine paradox." McDonald et al., (1997) reported the co-localization of the CAT-1 transporter and eNOS in caveolae of pulmonary artery endothelial cells, which would provide a

mechanism for the directed delivery of substrate to eNOS for NO synthesis (McDonald et al. 1997).

1.6 Hypotheses and Aims

Based on the evidence provided above, the goals of this project were to determine the effect of different shear rates on endothelial CAT-1, PKCα, and phosphorylated CAT-1 both with and without the addition of the uremic toxin urea. The overall hypothesis is that shear stress alters the expression of endothelial CAT-1 and CAT-1 phosphorylation state. Posch et al., (1999) showed that increases in shear stress resulted in increases in L-arginine uptake into the endothelium (Posch et al. 1999), which is known to occur through CAT-1 (Walther et al. 2004). However, in CKD rats that participated in wheel running, there was an increase in L-arginine transport that was not associated with an increase in CAT-1 expression. In both the sedentary and exercised rats, there were similar levels of CAT-1 expression, which was significantly lower than the SHAM group (Martens et al. 2014). It has been shown that CAT-1 activity is regulated by PKCα , possibly through phosphorylation, resulting in a decrease in its transport ability (Ingbir et al. 2008) Martens et al., (2014) also observed an increase in PKCα expression in sedentary animals compared to exercise animals, suggesting it acts on CAT-1 to mediate its transport activity.

1.6.1 Specific Aim 1: Determine the effect of different shear rates on endothelial CAT-1, PKCα, and phosphorylated CAT-1.

1.6.1.1 Hypothesis 1: Increasing levels of endothelial shear stress will result in an increase in total CAT-1 expression and a decrease in PKCα and phosphorylated CAT-1 expression.

1.6.2 Specific Aim 2: Determine the effect of different endothelial shear rates on CAT-1, PKCα, and phosphorylated CAT-1 expression in the presence of the uremic toxin urea.

1.6.2.1 Hypothesis 2: There will be no change in CAT-1 expression, and a decrease in PKCα and phosphorylated CAT-1 expression with increasing shear rates in the presence of the uremic toxin urea.

Chapter 2

MATERIALS AND METHODS

2.1 Materials

Single donor female human umbilical vein endothelial cells (HUVECs) purchased from Lonza (Walkersville, MD) were used. EGM-2 media and EGM SingleQuot were also purchased from Lonza. Urea used was from Sigma-Aldrich (St. Louis, MO). Trypsin-EDTA 0.25% was purchased from Sigma-Aldrich and was diluted in Hank's Balanced Salt Solution from Corning Incorporated (Corning, NY) to 0.05%. Fibronectin from bovine plasma was purchased from Sigma-Aldrich. The Flexcell streamer shear stress device used was from Flexcell International Corporation (Burlington, NC). Medium 199, 1X was purchased from Corning Incorporated. RIPA buffer and protease inhibitor cocktail mix used was from Thermo Fisher Scientific (Waltham, MA). Laemmli sample buffer was from Bio-Rad Laboratories (Hercules, CA). 10% Tris glycine gels were purchased from Thermo Fisher Scientific. Superblock T20 (TBS) blocking buffer was purchased from Thermo Fisher Scientific as well. CAT-1 and PKCα antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX). Phosphorylated CAT-1 antibody was purchased from Thermo Fisher Scientific. Secondary antibodies used were from Santa Cruz Biotechnology. WesternSure Luminol Enhance Solution and WesternSure Stable Peroxide Solution were purchased from LI-COR Biosciences (Lincoln, NE). C-Digit Blot Scanner was from LI-COR Biosciences. Image Digits Studio (Version 5.0) was also from LI-COR Biosciences.

2.2 Cell Culture

Human umbilical vein endothelial cells (HUVECs) (Lonza) were cultured in EGM-2 supplemented with EGM SingleQuot (Lonza) or in EGM-2 with 25mM urea (Sigma-Aldrich). Cells were maintained in a humidified incubator at 37°C with 5% CO2-95% air and passaged every 48-72 hours after reaching 70-100% confluency. Trypsin was used to detach cells from the flasks or microscope slides during cell splitting.

2.3 Shear Stress

Sterilized microscope slides were coated with fibronectin from bovine plasma (Sigma-Aldrich) and allowed to sit for 2-3 hours. After 6 passages, HUVECs were cultured on the slides in EGM-2 for 48 hours. When cells reached 100% confluency, shear stress was carried out in a Flexcell streamer shear stress device (Flexcell International Corporation) at 10, 15, and 20 dyn/cm^2 for 40 minutes using Medium 199, 1X (Corning Incorporated) as the flow medium.

2.4 Western Blotting

Total cell lysates were collected 24 hours after shear stress using RIPA buffer including a protease inhibitor cocktail mix (Thermo Fisher Scientific). Protein concentration was determined using a Bradford Protein Assay. Samples were diluted in Laemmli sample buffer (Bio-Rad Laboratories) containing β-mercaptoethanol and boiled for 5 minutes. Samples were loaded into 10% Tris glycine gels (Thermo Fisher Scientific) and electrophoresed for 60 minutes at 100 V. Gels were transferred to a nitrocellulose membrane, blocked with Superblock T20 (TBS) blocking buffer (Thermo Fisher Scientific) and immunoblotted with the primary antibody for the protein of interest: CAT-1 (1:200; Santa Cruz Biotechnology sc-66825), PKCα

(1:200; Santa Cruz Biotechnology sc-8393), or phosphorylated CAT-1, (1:200; Thermo Scientific PA5-12982). Membranes were washed and incubated with the appropriate recommended secondary antibody. Membranes were then incubated for 5 minutes in a substrate working solution containing WesternSure Luminol Enhance Solution (LI-COR 926-80020) and WesternSure Stable Peroxide Solution (LI-COR Biosciences 926-80020) and scanned on a C-Digit Blot Scanner (LI-COR; CDG-002561). Data are presented as intensity relative to β-actin (1:2,000; Santa Cruz Biotechnology sc-47778). Western blots were quantified using Image Digits Studio (Version 5.0, LI-COR).

2.5 Statistical Analysis

Data were analyzed using a one-way ANOVA and a Tukey's post hoc test was performed to assess differences between groups using GraphPad Prism 5.0 software. The alpha level was set at 0.05 and all data are presented as means ± SEM.

Chapter 3

RESULTS

3.1 Shear Stress Studies

When HUVECs were cultured in untreated media, CAT-1 expression increased as shear stress levels increased. CAT-1 expression at 20 dyn/cm^2 was significantly greater than at 0 dyn/cm^2 (Figure 3.1; p = 0.0329). PKCα expression decreased with increasing shear stress starting at 10 dyn/cm^2; however, this result was not significant (Figure 3.2; p = 0.767). Similarly, phosphorylated CAT-1 expression also decreased as shear stress increased starting at 10 dyn/cm^2; this result was not significant (Figure 3.3; p = 0.2194).

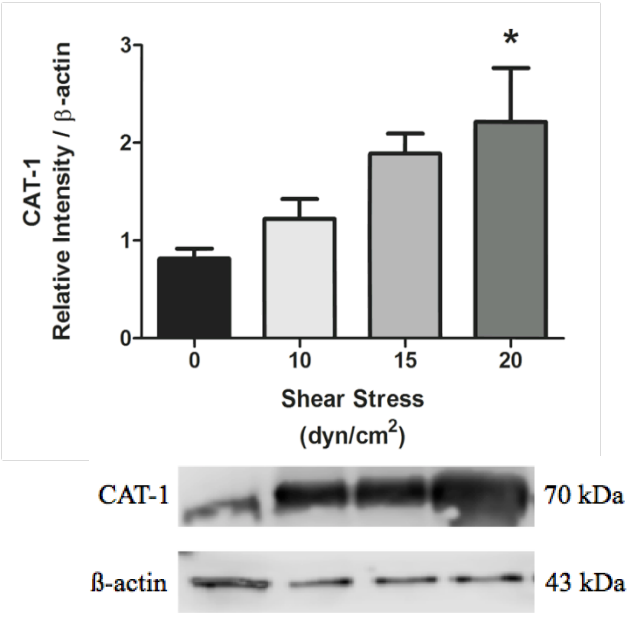

Figure 3.1 CAT-1 protein expression. CAT-1 intensity normalized to ß-actin measured by western blot. n = 4 in each group. *p < 0.05.

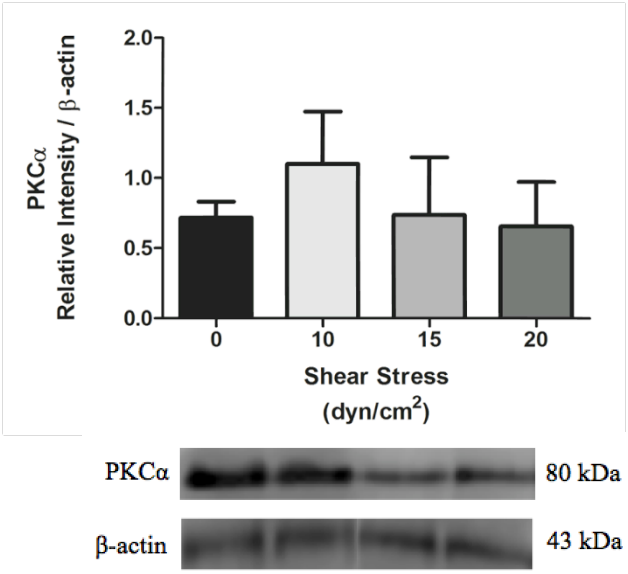

Figure 3.2 PKCα protein expression. PKCα intensity normalized to ß-actin measured by western blot. n = 4 in each group. Not significant.

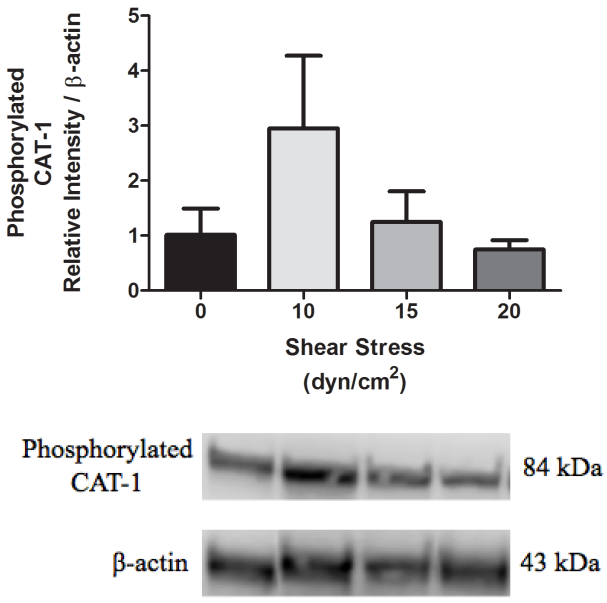

Figure 3.3 Phosphorylated CAT-1 expression. Phosphorylated CAT-1 intensity normalized to ß-actin measured by western blot. n = 4. Not significant.

3.2 Urea Studies

When HUVECs were cultured in 25mM urea, CAT-1 expression remained unchanged between the different shear stress levels (Figure 3.4 p = 0.9838). PKCα expression significantly decreased at all shear stress levels compared to 0 dyn/cm^2 (Figure 3.5 p = 0.0002). Phosphorylated CAT-1 expression was also significantly less at all shear stress levels compared to 0 dyn/cm^2 (Figure 3.6; p = 0.0002).

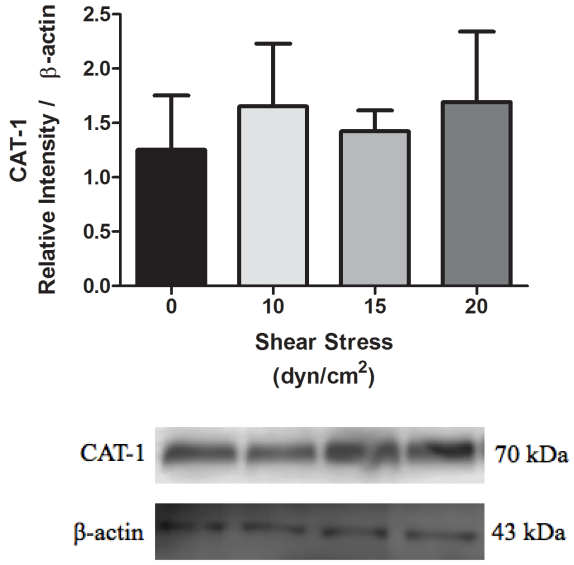

Figure 3.4 CAT-1 protein expression. CAT-1 intensity normalized to ß-actin measured by western blot. n = 3. Not significant.

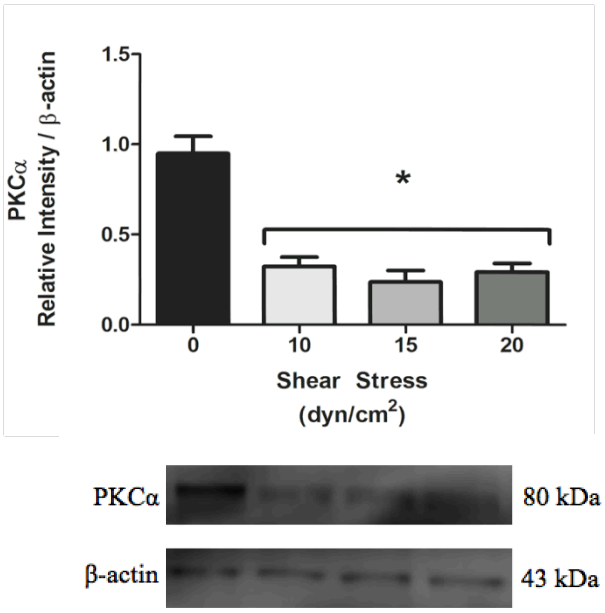

Figure 3.5 PKCα protein expression. PKCα intensity normalized to ß-actin measured by western blot. n = 3. *p < 0.05.

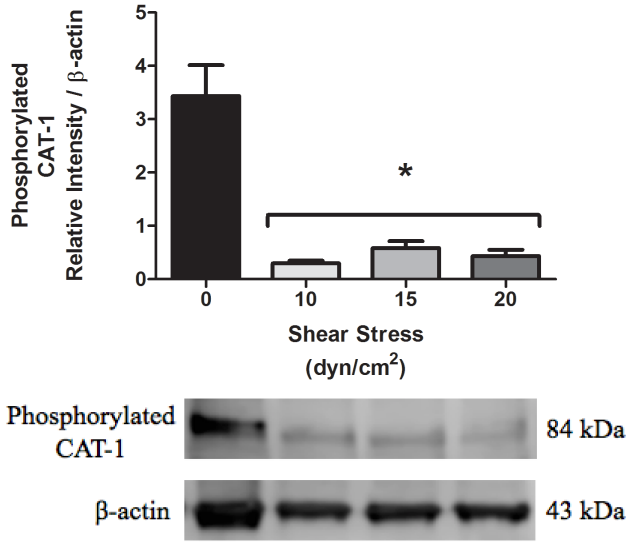

Figure 3.6 Phosphorylated CAT-1 expression. Phosphorylated CAT-1 intensity normalized to ß-actin measured by western blot. n = 3. *p < 0.05.

Chapter 4

DISCUSSION

The main finding of this study is that shear stress alters CAT-1 expression, as well as its expression in the phosphorylated state. In the presence of shear stress, CAT-1 protein expression increased as shear levels increased, while PKCα and phosphorylated CAT-1 protein expression decreased. Additionally, in the presence of 25mM urea, CAT-1 expression remained unchanged with increasing shear stress, and PKCα and phosphorylated CAT-1 expression was decreased compared to 0 dyn/cm^2.

Shear stress can improve vascular function through the release of nitric oxide via calcium dependent pathways. One of the ways that shear stress is detected by endothelial cells is through the glycocalyx, which then can initiate multiple signaling cascades, one of them involving intracellular calcium mobilization (Chistiakov et al. 2016). Shear stress leads to the generation of inositol 1,4,5 trisphosphate (IP3), which is associated with intracellular calcium mobilization, and diacylglycerol (DAG), which is linked to PKC activation, from membrane phosphatidylinositol (Ballerman et al. 1998). However, Malek et at., (1993) found that physiological shear stress did not significantly activate PKC (Malek et al. 1993). Future studies would need to be conducted in order to determine the mechanism by which shear stress alters PKCα activation.

Several studies have investigated the potential role of intracellular calcium mobilization in shear stress-mediated signaling on NO release. Macathur et al., (1993) found that thapsigargin, which depletes intracellular calcium stores, did not inhibit shear stress-mediated NO release. This suggests that NO release in response to shear

stress does not depend on mobilization of calcium from intracellular stores (Macarthur et al. 1993). However, shear stress-induced release is inhibited when extracellular calcium is removed, making it seem that NO release is partly dependent on calcium influx (Buga et al. 1991 and Xiao et al. 1997).

Increased shear stress has also been shown to be beneficial to vascular function through increased NO synthesis by way of increased L-arginine transport through CAT-1 (Weber et al. 2005), which is the primary method of transport for L-arginine movement into the endothelium (Closs 2006). Posch et al., (1999) reported that increases in shear stress resulted in increases in L-arginine transport in a stress-dependent manner in porcine aortic endothelial cells (Posch et al. 1999). Our results show that as shear stress was increased, CAT-1 expression also increased in a stress-dependent manner, with expression levels being significantly greater at 20 dyn/cm^2 compared to 0 dyn/cm^2 (Figure 3.1). This could provide a possible mechanism for the stress-dependent increase in L-arginine uptake that Posch et al., (1999) observed.

L-arginine transport via CAT-1 has been shown to be impaired in the presence of uremic plasma (Xiao et al. 2001) as well as in animal models of CKD (Schwartz et al. 2009 and Ingbir et al. 2008). This impairment is also accompanied by a decline in CAT-1 protein expression (Schwartz et al. 2009 and Martens et al. 2014). Shear stress resulted in an improvement in EDR in CKD rats through increased L-arginine uptake; however, this did not occur by way of increased CAT-1 expression. From this, we hypothesized that in the presence of 25mM urea, there would be no change in CAT-1 protein expression as shear levels increased, which is what we observed. This suggests that shear stress may mediate an increase in L-arginine uptake through a posttranslational modification of CAT-1 (Martens et al. 2014).

CAT-1 contains three sites for phosphorylation by PKCα (Rotmann et al. 2004), and these proteins have been shown to be localized in caveolae, which could allow for their interaction (Mineo et al. 1998). These findings provide evidence that PKCα is involved in regulating CAT-1 activity and more specifically, that PKCα regulation of CAT-1 leads to a reduction in L-arginine transport. Ingbir et al., (2008) reported that in uremic rats treated with rosiglitazone, L-arginine uptake was increased without an increase in CAT-1 expression. Treatment with rosiglitazone was associated with a decrease in PKCα, as well as a decrease in phosphorylated CAT-1 compared to untreated rats (Ingbir et al. 2008). Additionally, in rats with CKD that remained sedentary for 4 weeks, there was an increase in PKCα expression, while groups that participated in voluntary wheel running and were given oral L-arginine supplementation experienced normal expression levels (Martens et al. 2014). This increase in PKCα protein expression suggests that PKCα may be acting on CAT-1 and phosphorylating it, ultimately leading to an overall decline in L-arginine transport into the endothelium. In the present study, we observed a decrease in both PKCα protein expression levels and phosphorylated CAT-1 protein expression levels as shear stress was increased starting at 10 dyn/cm^2 (Figure 3.2 and Figure 3.3). At 0 dyn/cm^2, there is a decline in both PKCα and phosphorylated CAT-1 compared to 10 dyn/cm^2. This could simply be due to normal inhibition of the expression of these proteins since 0 dyn/cm^2 is well below the physiological level of shear stress of approximately 10-12 dyn/cm^2. Furthermore, we found that in the presence of the uremic toxin, urea, at a concentration of 25 mM, there was a significant decline in PKCα protein expression and phosphorylated CAT-1 protein expression at each shear stress rate compared to 0 dyn/cm^2 (Figure 3.5 and Figure 3.6). However, additional research would need to be

done in order to determine the mechanism by which shear stress causes a decrease in PKCα expression.

The exact mechanism by which PKCα acts on CAT-1 and causes an attenuation in L-arginine uptake is not fully understood. Krotova et al., (2003) found that the activation of PKCα with PMA reduced L-arginine uptake in pulmonary artery endothelial cells without changing the expression or subcellular distribution of CAT-1. PMA activation of PKCα has previously been shown to result in its translocation to plasma membrane caveolae, which is where CAT-1 is localized (Krotova et al. 2003). This suggests that the translocation of PKCα to membrane caveolae induces phosphorylation of CAT-1, resulting in inhibition of L-arginine transport. Depletion of PKCα was shown to promote dephosphorylation of CAT-1 as well as activation of transport activity. Here, it is likely that PKCα directly regulates CAT-1 transport activity (Krotova et al. 2003). In another study also conducted on pulmonary artery endothelial cells, PMA activation of PKCα resulted in the internalization of CAT-1 to the cytosol of *Xenopus laevis* oocytes independently of CAT-1 phosphorylation, which was accompanied by a similar reduction in L-arginine uptake (Rotmann et al. 2004). In this case, it would seem likely that the transport inhibition by PKCα is due to the translocation of CAT-1 to the cytosol, rather than modifications in CAT-1 catalytic activity (Rotmann et al. 2004). Whether PKCα mediates a decrease in L-arginine transport through a direct reduction in CAT-1 catalytic activity or by the internalization of CAT-1 in CKD remains unclear. In our experiments, as evidenced by a significant decrease in PKCα expression and phosphorylated CAT-1 expression at increased shear rates, it seems likely that shear stress may cause a direct alteration in CAT-1 catalytic activity, which could ultimately effect L-arginine transport.

However future studies would be necessary in order to determine whether or not PKCα also causes CAT-1 translocation to the cytosol of the cell.

These findings can also be related to exercise, as increased shear stress occurs during the increase in blood flow that is associated with exercise. Exercise has been shown to improve vascular function through the L-arginine transport and increased NO synthesis (Sitia et al. 2010). For example, it has been found that short-term daily exercise activity significantly augmented the dilation of skeletal muscle arterioles in rats in response to L-arginine through an enhanced endothelial synthesis of NO (Sun et al. 2016). It is possible that this observation could be explained by an increase in L-arginine transport via an increase in CAT-1 expression, which is consistent with our findings that CAT-1 expression increases with increased shear stress. Additionally, 4 weeks of voluntary wheel running in rats with CKD resulted in a significant increase of L-arginine uptake and vascular function (Martens et al. 2014). However, this improvement in L-arginine uptake was not accompanied by an increase in CAT-1 expression. Interestingly, there was a decrease in CAT-1 protein expression in CKD rats compared to SHAM animals (Martens et al. 2014). We observed similar results because in the presence of 25 mM urea, there was no change in CAT-1 protein expression as shear levels increased. This would suggest that in CKD, exercise may mediate an increase in L-arginine uptake through a post translational modification of CAT-1 (Martens et al. 2014).

It has been suggested that PKCα is involved in the regulation of CAT-1 activity (Ingbir et al. 2008 and Rotmann et al. 2004). In our experiments in the presence of 25 mM urea, there was a significant decline in both PKCα and phosphorylated CAT-1 as shear levels increased, suggesting that PKCα alters the

catalytic activity of CAT-1 through phosphorylation. Furthermore, in rats with CKD that remained sedentary for 4 weeks, there was an increase in PKCα expression, while groups that participated in voluntary wheel running and were given oral L-arginine supplementation experienced normal expression levels. The normal expression level of PKCα in these animals was accompanied by a significant increase in L-arginine uptake, which would suggest that exercise influences L-arginine transport through the alteration of CAT-1 catalytic activity and can ultimately be very beneficial in improving vascular function in CKD (Martens et al. 2014).

In conclusion, our findings have shown that shear stress may be advantageous in the treatment of CKD. In the presence of urea, increased shear stress did not increase CAT-1 expression, signifying a potential posttranslational effect on CAT-1; however it did decrease both PKCα and phosphorylated CAT-1 expression. If PKCα expression is decreased and in turn not inhibiting CAT-1 transport activity, this could provide a mechanism for enhanced L-arginine uptake into the endothelium. This would lead to additional NO synthesis and vasorelaxation, which is why shear stress by way of exercise could be an effective strategy in the treatment of CKD to improve vascular health.

4.1 Future Directions

Our study has provided valuable insight into how shear stress affects the expression levels of certain proteins that are involved in vascular function. Also, it has offered a possible explanation for the improvement of L-arginine uptake and vascular function resulting from exercise that has been reported in several previous studies. Our findings emphasize the importance of CAT-1 on vascular function, and

that shear stress can be beneficial in improving vascular function thorough the regulation of certain protein expression levels. These findings have also demonstrated the potential benefit of exercise on vascular function in CKD thorough the regulation of CAT-1, PKCα, and phosphorylated CAT-1 expression, as well as offered a possible treatment for the endothelial dysfunction in CKD through enhanced L-arginine transport. The main experiment that future studies should include would be L-arginine uptake assays using a scintillation counter in order to determine how shear stress and the expression levels of CAT-1, PKCα, and phosphorylated CAT-1 affect L-arginine transport into cells. It would also be important to include experiments to determine how PKCα regulates CAT-1 in more detail. We have seen that PKCα acts on CAT-1 and phosphorylates it, ultimately altering its catalytic activity. However, it is still not known if this mechanism of regulation includes the translocation of CAT-1 into the cytosol of the cell, or if CAT-1 remains bound to the plasma membrane. These experiments could be accomplished through the technique of immunohistochemistry. Finally, future experiments should also include using other cell types, such as pulmonary artery endothelial cells or bovine or porcine aortic endothelial cells. It would be interesting to see if similar results were obtained from different cell types than human umbilical vein endothelial cells.

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