IMPACT OF EXERCISE ON RENAL NITRIC OXIDE AND ANTIOXIDANT SYSTEMS

By

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To my Opa (grandfather), Pinardi Rorong

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Exercise has numerous cardiovascular benefits including increased blood flow which increases shear stress leading to induction of the endothelial nitric oxide (NO) synthesizing enzyme (eNOS). The antioxidant, extracellular superoxide dismutase (EC SOD) is also induced and both enzymes are required for optimal vascular health. In the kidney, however, exercise reduces blood flow, which might lead to falls in NO and antioxidant bioavailability. This dissertation focuses on the impact of exercise on renal NO and antioxidants in health and in injury. In the cardiovascular injury prone Sprague Dawley (SD) rat, low-intensity voluntary exercise reduced renal eNOS and EC SOD, and this was associated with increased susceptibility to acute kidney injury (AKI) induced by ischemia/reperfusion (IR). In contrast, Fisher 344 (F344) rats, a strain resistant to cardiovascular injury, voluntary exercise increased renal eNOS/EC SOD in association with protection against AKI. Real-time assessment of renal blood flow (RBF) revealed that exercise training reduces resting RBF and that the fall in RBF seen with acute exercise in sedentary rats is blunted by training in both strains. Studies in the aging F344 revealed that chronic treadmill exercise did not alter age-related renal injury. We also confirmed that protection against age-related renal injury is associated

with a preserved renal NO system in the “protected” F344xBrown Norway. We have not uncovered the mechanism for the different renal eNOS/EC SOD responses to exercise in the SD vs. F344. However, these data indicate that the renal response to exercise and age are influenced by genetic background and that exercise influences the status of pre-existing renal endothelial health which determines the severity of IR-induced AKI. Consideration of these factors is required for optimal exercise benefit for patients of endothelial dysfunction including but not limited to renal disease.

CHAPTER 1
INTRODUCTION

According to the 2010 annual report by the US Renal Data System, renal failure afflicts an estimated 11.5% of the adult population and creates a tremendous economic burden on the healthcare system. These numbers are predicted to increase as a result of the rising incidence of cardiovascular disease, a co-morbidity of chronic kidney disease (CKD). Advancing age also increases the risk of developing renal failure, a concern since the aging population continues to grow.

Lifestyle modifications involving physical activity have proven effective in reducing the risk for cardiovascular mortality which is high in patients with renal failure. Metabolic benefits of exercise include reductions in plasma triglycerides, increases in the high-density lipoprotein to low-density lipoprotein ratio, and improved insulin sensitivity and overall cardiac function (Sasaki & Gisele, 2005). Also important are the direct vascular effects of exercise. In vascular beds where blood flow increases during exercise, shear stress directly interacts with the endothelium to up-regulate essential factors required for optimal endothelial health. However, in the kidney, blood flow is reduced during exercise and few have thoroughly investigated exercise-induced endothelial adaptations. Indeed, there are reports of exercise-induced acute kidney injury (AKI) in man (Seedat *et al.* 1990; Yan *et al.* 2010; Bosch *et al.* 2009) and exercise-induced exacerbation of age-associated renal structural injury in mice (Lichtig *et al*. 1987). Certainly, the impact of exercise on the aging kidney and on the susceptibility to AKI is of considerable clinical relevance. The purpose of this section is to provide background regarding the cardiovascular and renal responses to exercise, with an emphasis on exercise-induced endothelial adaptations in both circulations. It

will also explore literature pertaining to the impact of exercise on the kidney in the presence of underlying renal injury, and will end with the objectives of this work.

Cardiovascular Responses to Exercise

Cardiovascular Adaptations to Exercise

Exercise stresses the regulatory ability of the cardiovascular system. It increases heart rate, stroke volume, and the total systemic arteriovenous oxygen difference. The ability for each of these variables to increase determines maximal oxygen uptake (VO_2max), the functional capacity of the cardiovascular system. Oxygen uptake and exercise intensity increase linearly but eventually oxygen uptake plateaus despite further increases in exercise intensity (Rowell, 1993). Thus, VO_2max is attained at submaximal rates of exercise intensity. In normal young individuals, VO_2max ranges from 45 to 53 mL/kg/min and up to 85 mL/kg/min in endurance athletes (Rowell, 1993).

Cardiac function improves as a consequence of physical conditioning, a term to describe repeated exposure to exercise training. Part of this adaptation involves greater cardiac output mainly due to increases in stroke volume and oxygen extraction despite a decrease in heart rate. While some studies report the requirement of both mechanisms, others demonstrate that greater maximal cardiac output can occur with minimal increases in oxygen extraction (Clausen *et al.* 1977; Saltin *et al.* 1969). Improvements in stroke volume are mainly due to changes that enhance ventricular contraction as dictated by the Frank-Starling law of the heart: End diastolic volume increases due to a combination of increased blood volume, thickening of the left ventricular wall, quicker ventricular filling, and increased myocardial contractility (Rowell, 1993). Improvements in oxygen extraction are partly dictated by the regional vasoconstriction of the renal and splanchnic circulation. Shunting of blood from these

vascular beds provides greater blood supply to active muscles. In addition, in the muscle where metabolic demand is high during exercise, oxygen extraction increases due to increased skeletal muscle vascular conductance which provides greater capillary blood volume. In turn, this reduces diffusion distance between the microcirculation and the muscle fiber, resulting in increased efficiency of oxygen delivery (Rowell, 1993).

Mechanisms of vascular conductance will be described in greater detail in the section entitled '*Endothelial adaptations to exercise: The role of nitric oxide'.*

Physical conditioning also reduces cardiovascular risk factors. It decreases blood pressure, body fat, plasma triglycerides, total cholesterol, and increases insulin sensitivity (Sasaki & Gisele Dos Santos, 2005). Beneficial metabolic actions of exercise have been proven effective in treatment of hypertension (Fagard, 2011), diabetes (Nishida *et al.* 2004), and obesity (Savage *et al.* 2004). Finally, the benefits of physical conditioning extend to the endothelium.

Endothelial Adaptations to Exercise: The Role of Nitric Oxide

A large body of literature supports efficacy of exercise training in improving endothelial function via a nitric oxide (NO)-mediated mechanism (Delp *et al.* 1993; Delp and Laughlin, 1997; McAllister *et al.* 2009; Sindler *et al.* 2009; Mora *et al.* 2007; Green *et al.* 2004). Using the inhibitor of NO formation, N^G-nitro-L-arginine methyl ester (L-NAME), Delp *et al.* demonstrated that enhanced acetylcholine-mediated dilation with training was due to an increase in NO production (Delp *et al.* 1993). In rats, this response is present by four weeks of endurance treadmill training (Delp & Laughlin, 1997).

Nitric oxide (NO) is an essential signaling molecule that regulates vasomotor tone (Ignarro *et al.* 1987) and protects the endothelium against development of

atherosclerotic lesions via inhibition of lipid oxidation, vascular smooth muscle cell proliferation and platelet aggregation (Harrison *et al.* 2006). NO is produced by NO synthase (NOS) using the substrates L-Arginine and oxygen. Three NOS isoforms exist: endothelial (e) NOS, neuronal (n) NOS, and inducible (i) NOS. All require the cofactors tetrahydrobiopterin (BH_4), calmodulin (CaM), flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN).

Shear stress, or the frictional force exerted on a vessel wall caused by blood flow, stimulates production of nitric oxide (NO). Within seconds, shear stress increases intracellular calcium levels resulting in binding of calmodulin to eNOS, thus resulting in eNOS activation (Harrison *et al.* 2006). In addition, protein kinase A phosphorylates eNOS on serines 635 and 1177 after the onset of shear, resulting in increased enzyme activity. In situations of prolonged shear stress, eNOS mRNA stability increases (Davis *et al.* 2001). This in turn increases eNOS transcription and translation. Shear stress also up-regulates the expression of the extracellular superoxide dismutase (EC SOD) (Fukai *et al.* 2000), an antioxidant that protects against oxidative stress by scavenging for the highly reactive superoxide molecule. Two other SOD isoforms have been characterized, the cytosolic CuZn SOD and mitochondrial Mn SOD; however, EC SOD is the major superoxide scavenger in the vascular extracellular space (Harrison *et al.* 2006). Mice deficient in c-Src, a tyrosine kinase required for the shear stress response, failed to increase their aortic eNOS and EC SOD protein levels with exercise training (Davis *et al.* 2003), confirming the role of blood flow and shear stress in exercise-induced endothelial adaptations. Furthermore, eNOS knock-out mice failed to show increases in EC SOD, despite exercise training, suggesting that increased NO is

required for EC SOD up-regulation (Fukai *et al.* 2000). Thus, increased shear stress improves endothelial function in circulations where exercise increases blood flow. However, little is known about the impact of exercise training in areas where blood flow is reduced.

Renal Responses to Exercise

Exercise Reduces Renal Blood Flow

In contrast to other vascular beds, exercise reduces renal blood flow (RBF). Peripheral vasoconstriction in the renal and splanchnic circulations is required to supply working muscles with increased blood flow. In humans, falls in RBF and renal plasma flow (RPF) occur immediately and are dependent on exercise intensity (Poortmans 1985). Paraminohippurate (PAH) clearance measured in healthy young men revealed dose-dependent falls in RPF with increasing exercise intensity and that the declines remained even after 40 minutes of recovery following exercise (Chapman *et al.* 1948). Using radioactive microspheres, exercise-trained miniature swine reduced their RBF (Bloor *et al.* 1986). In the dog, while some studies report a reduction in RBF with exercise (Delgado *et al.* 1975; Musch *et al.* 1987), others find the opposite response (Sadowski *et al.* 1981). It is likely that the dog is relatively resistant to exercise-induced falls in RBF but nevertheless exhibit declines at severe intensity. Clearly, species and possibly strain differences are present. Despite exercise-induced falls in RBF in human and swine, glomerular filtration rate (GFR) is relatively well-maintained at low and moderate exercise intensities due to increases in filtration fraction (FF) (Castenfors, 1967). However, at high intensities (>50-60% VO_2max), GFR declines (Merrill & Cargill 1948; Kachadorian & Johnson, 1970).

Various mechanisms mediate exercise-induced falls in RBF including increased sympathetic nervous outflow and the participation of several hormone systems. Renal sympathetic nerve activity increases as a function of exercise intensity (O'Hagan *et al.* 1993). In the denervated rabbit kidney, exercise failed to cause renal vasoconstriction and a fall in RBF, confirming the participation of the renal nerves (Mueller *et al.* 1998). Angiotensin II, endothelin-1, and vasopressin also mediate the decline in RBF during exercise (Stebbins *et al.* 1995, Ahlborg *et al.* 1995; Maeda *et al.* 2004; Stebbins *et al.* 1993). Indeed, plasma renin activity increases due to increases in renal nerve activity (Lifschitz & Horwitz, 1976). In man, there is also a role for prostaglandins since the non-selective cyclooxygenase-2 inhibitor indomethacin exaggerated the reduction in RBF after 30 minutes of strenuous exercise (Walker *et al*. 1994). Thus, several mechanisms are involved in the reduction of RBF during exercise.

Exercise Reduces Urinary Sodium Excretion

Renal handling of sodium is crucial in establishing total body fluid homeostasis, especially during exercise where volume depletion due to water and sodium loss through sweating occurs. In an attempt to conserve sodium, free water clearance and urinary sodium excretion significantly falls with exercise (Baker *et al.* 2005). This response is primarily due to increased tubular reabsorption (Castenfors, 1977). Interestingly, a decrease in urinary sodium excretion is not entirely attributable to hormones of the renin-angiotensin system since pharmacological inhibition of angiotensin II had no effect on the anti-natriuretic response of exercise (Mittelman, 1996; Wade, 1987). Treatment with an aldosterone antagonist also failed to alter the decrease the urinary sodium excretion with exercise (Zambraski, 1990). However, these findings may reflect an issue with dosage. Future studies are required to define

the role of the renin-angiotensin system in the response of reduced urinary sodium excretion with exercise. It is likely that the exercise-induced renal sodium retention may also reflect increased sympathetic nerve activity to directly enhance tubular sodium reabsorption.

Exercise-Induced Proteinuria

Another renal response of exercise training is the appearance of proteins in the urine. Although proteinuria is a hallmark of renal injury, exercise-induced proteinuria is transient, lasting for only 24 hours after exercise, and is considered normal (Coye & Rosandich, 1960). Various urinary proteins have been identified including albumin, transferrin, ceruloplasmin, and immunoglobulin G (Rowe & Soothill, 1961). As with falls in RBF, post-exercise proteinuria is directly related to exercise intensity and may be secondary to increased glomerular permeability and/or reduced proximal tubular protein reabsorption (Poortmans & Labilloy, 1988; Poortmans & Vanderstraten, 1994; Poortmans, 1990). Urinary excretion of heparan sulfate proteoglycan, components of the glomerular basement membrane responsible for anionic sites, significantly increased from 24±8 to 235±77 ng/min after 45 minutes of mild bicycle exercise in normotensive subjects (Heintz *et al.* 1995). Furthermore, intravenous injections of the NADPH oxidase inhibitor, diphenyleneiodonium chloride, four days before exercise, attenuated exercise-induced increases in urinary excretion rates of protein and the oxidative stress markers, thiobarbituric acid-reactive substance and protein carbonyl contents (Kocer *et al.* 2008). These studies suggest that causes of increased glomerular permeability include transient loss of glomerular charge and increased oxidative stress (Fox JG *et al.* 1993; Poortmans & Vanderstraten, 1994). Mittleman et al. also showed that indomethacin ameliorated the urinary excretion of protein after 30

minutes of strenuous exercise in human subjects (Mittelman *et al.* 1992), suggesting a direct or indirect role for prostaglandins. Since exercise-induced proteinuria occurs in the normal kidney, it will be crucial to determine the compounding impact of renal disease.

Renal Adaptations to Chronic Exercise

Chronic exercise results in an attenuation of renal vasoconstriction and therefore RBF during acute exercise. Using the microsphere technique to determine regional blood flows in rats, Armstrong and Laughlin demonstrated that chronic exercise reduced the magnitude of the decrease in RBF after treadmill-exercise (Armstrong & Laughlin, 1984). These adaptations were despite differences in baseline values between trained and untrained groups. The study also reported that exercise training led to blunted falls with acute exercise in blood flows to organs of the splanchnic circulation (i.e. spleen, liver, stomach, duodenum, and colon). This adaptation is likely due to a combination of training-induced reductions in sympathetic outflow, angiotensin II, vasopressin, and norepinephrine (McAllister, 1998). The type of exercise training also plays a role since in rats endurance treadmill confers this adaptation but not high-intensity sprint training (Musch *et al.* 1996).

Although less understood, local endothelial changes may also contribute to the reduced renal vasoconstrictor response to exercise in the trained setting. In isolated perfused kidneys from New Zealand White rabbits, De Moraes et al. showed that 12 weeks of treadmill exercise enhanced acetylcholine-induced vasodilation and that L-NAME blunted this response, suggesting that the adaptation of exercise training was due to increased NO bioavailability (De Moraes *et al.* 2004). However, studies from Miyauchi et al. in rats suggest otherwise. These investigators observed a reduction in

renal eNOS mRNA, protein, and enzyme activity after acute treadmill exercise, whereas in the lung these measurements increased (Miyauchi *et al.* 2003). Their findings argue that exercise reduces shear stress in the kidney and that in organs where blood flow is increased with exercise, increased shear stress follows, resulting in NO up-regulation. On the other hand, Padilla *et al.* postulate that despite reductions in RBF during exercise, the combination of increased cardiac output and renal vasoconstriction may lead to increases in shear stress and therefore increases in NO production (Padilla *et al.* 2011). To date, the levels of shear stress in the renal circulation during exercise have not been measured and will be extremely difficult to determine in vivo given the complexity of the renal circulation (Moffat & Fourman, 1963). Thus, renal endothelial adaptations in response to exercise training require further study. In addition, it will be important to determine how exercise influences development of kidney disease.

Exercise and Renal Disease

Chronic Kidney Disease and NO Deficiency

Nitric oxide (NO) deficiency contributes to the progression of chronic kidney disease. Several mechanisms cause NO deficiency including decreased protein abundance of the NO synthesizing enzyme, NO synthase (NOS), deficiency of the NOS substrate, L-arginine, and increased levels of the endogenous NOS inhibitor, asymmetric dimethylarginine (ADMA) (Baylis, 2008). A reduction in total NO production, as measured by urinary nitrite and nitrate (stable metabolites of NO) levels, is evident in various rodent models of CKD including the 5/6 ablation/infarction, chronic glomerulonephritis, chronic puromycin aminonucleoside nephrosis, and the aging rat (Baylis 2009). Moreover, chronic NOS inhibition leads to hypertension, proteinuria, and structural injury in the form of glomerular sclerosis, tubulointerstitial injury, and

glomerular ischemia (Zatz & Baylis, 1998). Oxidative stress as a result of increased reactive oxygen species and decreased antioxidants can also reduce NO bioavailability, thus further exacerbating renal injury. In the kidney, superoxide is mainly generated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and reacts with NO to form peroxynitrite, a highly reactive nitrogen species involved in lipid peroxidation (Gill & Wilcox 2006). Increases in renal p22phox, a subunit of NADPH oxidase, up-regulates NADPH oxidase activity, leading to oxidative stress (Chabrashvili *et al.* 2003*)*. Indeed, p22phox silencing prevents oxidative stress in the angiotensin-II induced model of hypertension (Modlinger *et al.* 2006). Additional studies have also shown increased oxidative stress in CKD (Vaziri, 2004; Dounousi *et al.* 2006).

Oxidative stress also mediates development of acute kidney injury (AKI). The most common cause of AKI is an ischemic insult followed by reperfusion. This leads to recruitment of local inflammatory signals which up-regulate reactive oxygen species resulting in reductions in NO bioavailability and therefore development of renal dysfunction (Le Dorze *et al.* 2009). Morphological changes accompanied with ischemia/reperfusion-induced AKI include disruption of cell-cell junctions, endothelial cell swelling, and alterations in the glyocalyx proteins of the renal endothelium (Sutton *et al.* 2003). Thus, endothelial injury characterized by increased oxidative stress and reduced NO bioavailability is the main mechanism by which ischemia/reperfusion induced AKI propagates.

Protection afforded against development of renal injury associates with having a maintained NO system and genetic background plays a role. For example, in the Wistar-Furth rat, resistance to CKD-induced by puromycin administration parallels with

preserved NO production (Erdely *et al*. 2004). Furthermore, minimal age-related renal injury in the Fisher 344XBrown Norway rat associates with increased renal NOS protein abundance (Moningka *et al.* 2011). In contrast, in the Sprague Dawley rat, development of significant renal injury after 11 weeks of 5/6 ablation/infarction is accompanied with falls in renal and total NO production (Erdely *et al.* 2003). Male gender is another risk factor since female Sprague Dawley rats exhibit maintained renal NOS protein abundance with age and are protected against age-dependent kidney damage compared to males (Erdely *et al.* 2003; Baylis 2009). The sexual dimorphism seen with NO deficiency as it relates to aging is presumably due to sex hormones. Thus, genetic background influences the progressive renal injury related to renal NOS deficiency, with females and rat strains such as the Wistar-Furth and Fisher 344xBrown Norway protected.

The Aging Kidney: A Model of CKD

The aging kidney is a model of slowly developing CKD and is also associated with NO deficiency. Although not inevitable, GFR declines with age, and more rapidly in males compared to females (Lindeman *et al.* 1985; Wesson, 1969). Falls in GFR are due to structural damage of the renal blood vessels caused, in part, by increased extracellular matrix accumulation and expansion of the glomerular mesangium (Leon *et al.* 2003). This leads to development of glomerular sclerosis and ischemia, and tubulointerstitial injury (Neugarten *et al.* 1999; Thomas *et al.* 1998). The culmination of structural injury eventually results in loss of functioning nephrons. Functional declines are due to increased renal vasoconstriction and falls in RPF (Baylis, 2009). In aged rats, micropuncture studies revealed increased glomerular pressure due to a loss in afferent arteriolar resistance (Anderson *et al.* 1994). A sustained increase in glomerular

pressure eventually leads to glomerular injury (Brenner *et al.* 1996). However, age-related declines in renal function and structural injury can occur without development of glomerular hypertension (Remuzzi *et al.* 1988; Baylis, 1994).

A mechanism of age-related renal injury is endothelial dysfunction, mainly a result of NO deficiency. Causes of NO deficiency are similar to those in CKD: loss of NOS protein abundance/activity, NOS substrate deficiency, oxidative stress and increased presence of endogenous NOS inhibitors (Baylis *et al.* 2009). Furthermore, several cardiovascular risk factors can accelerate the rate of age-related loss of renal function including male gender, hypertension, dyslipidemia, and accumulation of advanced glycosylation end products (Weinstein & Anderson, 2010).

Exercise and Chronic Kidney Disease

In the clinical setting, there are both risks and benefits with physical exercise in CKD. Risks include renal ischemia, dysrhythmia, and musculoskeletal injury but these are usually associated with high intensity exercise (Johansen, 2007). There are numerous benefits including improved blood pressure control, mental health, and muscle strength (Johansen, 2007). However, in patients with established CKD, exercise has been reported as of no benefit in slowing down the rate of decline in renal function (Eidemak *et al.* 1997). Concern has been expressed that because of the transient post-exercise proteinuria, exercise might actually worsen the rate of progression with established CKD (Poortmans & Vanderstraeten, 1994). Several rat studies suggest that the cardiovascular benefit of exercise does not necessarily guarantee renal benefit in the setting of CKD where both cardiovascular and renal complications are present. Voluntary exercise effectively ameliorated the increase in several cardiac proinflammatory markers associated with CKD in the female rat;

however, the study did not assess these markers in renal tissue (Bai *et al.* 2009). Using the renal mass reduction model of chronic kidney disease (CKD), Heifets et al. reported that exercise was beneficial since it increased GFR and reduced proteinuria and the level of glomerular sclerosis (Heifets *et al.* 1991). In contrast, Adams et al. found that voluntary exercise did not ameliorate the hypertension associated with the 5/6 nephrectomy model (Adams *et al.* 2004). In the same CKD model, Bergamaschi et al. reported that 60 days of treadmill exercise did not prevent CKD-induced proteinuria and glomerular slcerosis despite normalization of hypertension (Bergamaschi *et al.* 1997). Others have also demonstrated both renoprotective and anti-hypertensive effects with exercise in rat CKD models (Lu *et al.* 2006; Kanazawa *et al.* 2006). Therefore, it remains uncertain whether exercise training can reverse progression of CKD.

A key factor in the renal response to exercise relates to the impact on the renal eNOS and ECSOD since intrarenal NO-deficiency is associated with both experimentally induced and age-dependent CKD. The findings by Miyuachi et al. that exercise reduces renal eNOS in normal rats raises the possibility that the NO-deficiency associated with CKD could be exacerbated by exercise (Miyauchi *et al.* 2003). Exhaustive exercise in the young rat also decreased renal cortical NOS and SOD activity (Lin *et al.* 2010). Furthermore, four weeks of treadmill exercise worsened renal injury in rats with chronic NOS inhibition (Kuru *et al.* 2005). Despite a blood pressure lowering effect, exercise magnified the arteriolar wall thickening, focal tubular atrophy, and interstitial inflammatory infiltration associated with chronic NOS inhibition. This is of particular concern in the aging kidney where falls in eNOS abundance and NOS activity (Erdely *et al*. 2003) and increased oxidative stress occur (Gomes *et al.*

2009) in conjunction with slowly developing glomerular and tubulointerstitial injury (Baylis and Corman,1998). Additional deficits in renal NO due to exercise could exacerbate renal age-dependent injury. Indeed, six weeks of exercise in old C57BL/6J mice magnified the age-associated renal structural injury (Lichtig *et al*. 1987). Poortmans and Ouchinsky sought to determine the impact of maximal exercise on post-exercise proteinuria in aging man. Their findings suggest no harmful effect of exercise training on urine albumin excretion in the elderly; however, aged participants had no past history chronic disease of any type, smoking, or evidence of kidney/liver dysfunction so were considered a relatively healthy elderly population (Poortmans & Ouchinsky, 2006). It is likely that genetic background plays a role in dictating the renal response to exercise, whether in CKD or aging.

Exercise and Acute Kidney Injury

Even in the absence of pre-existing injury, renal injury due to exercise has been reported. Exercise-induced AKI reflects renal ischemia caused by rhabdomyolysis (the breakdown of muscle fibers) and/or severe volume depletion (Seedat *et al.* 1990; Yan *et al.* 2010; Bosch *et al.* 2009). Cases are usually associated with high intensity exercise such as marathon running (Clarkson, 2007). The increased oxidative stress accompanied by reperfusion injury following ischemia is likely to contribute to exercise-induced AKI. Furthermore, there is likely to be even greater susceptibility to renal injury by exercise if pre-existing oxidative injury exists as in the case of patients with renal hypouricemia, a condition where uric acid, an antioxidant, is low in plasma. Indeed, these patients frequently develop exercise-induced acute renal failure (Yan *et al.* 2010; Saito *et al.* 2011; Ishikawa, 2002). Together, these studies suggest that exercise may

increase susceptibility to an oxidative stress-mediated insult such as ischemia/reperfusion induced acute renal failure.

Summary and Objectives

Exercise improves cardiovascular function and provides benefit in several diseases including hypertension, diabetes, and obesity. One important mechanism by which exercise benefits the vasculature is through shear stress, the frictional force of blood flow on the endothelium. During exercise, shear stress increases as a result of increased blood flow to some parts of the circulation. This stimulates production of NO which has vasodilatory and anti-atherosclerotic properties and EC SOD which is an antioxidant that protects against oxidative stress. Several studies have characterized the benefits of exercise-induced endothelial adaptations but mainly in hyperemic tissue during exercise (i.e. skeletal muscle). Few, however, have thoroughly studied the impact of exercise on the kidney. Overall renal function declines during exercise since blood flow and GFR are reduced due to renal vasoconstriction. Exercise also leads to transient proteinuria, a hallmark of renal injury. In the literature, efficacy of exercise training in CKD and on age-related kidney damage is controversial. Furthermore, studies by Miyauchi et al.demonstrate that acute exercise decreases renal NO production in the healthy, normal rat, presumably due to falls in renal shear stress (Miyauchi *et al.* 2003). This is of particular concern since NO deficiency is a cause and consequence of CKD and age-related renal injury. Therefore, the following studies were conducted to determine impact of exercise on the renal NO and antioxidant systems in the normal, healthy kidney and in models of the acute and chronic kidney injury (i.e. aging). This dissertation presents the work in four separate chapters which are listed below with their respective objectives.

To characterize the impact of chronic voluntary exercise on renal cortical eNOS and EC SOD protein abundance. We determined whether duration of training influences the renal endothelial responses to 6 and 12 weeks of voluntary exercise in two different strains with differing cardiovascular risk profiles; the Sprague Dawley (SD) rat which is susceptible to experimentally-induced hypertension and the Fisher 344 (F344) which is resistant.

To characterize the impact of chronic voluntary exercise on susceptibility to acute renal injury. We determined whether chronic voluntary exercise exacerbates susceptibility to ischemia/reperfusion induced acute renal failure. To determine the role of genetic background, we compared the SD and F344. We also used the radiolabeled-microsphere technique to detect strain difference responses in renal blood flow during exercise.

To characterize impact of chronic treadmill exercise on progression of age-dependent renal injury. We determined whether chronic treadmill exercise exacerbates age-dependent renal injury in the male F344 rat. We assessed the renal NOS and SOD enzymes as well as the role of oxidative stress.

To determine if protection against age-dependent renal injury in the Fisher 344xBrown Norway rat is associated with maintained renal nitric oxide synthase. To further examine the role of genetic background on renal NOS, we investigated the impact of aging on various determinants of NO production in the F344xBrown Norway rat, a model of healthy aging. We also investigated whether there would be any beneficial effect of chronic renin-angiotensin system (RAS) blockade in these “protected” rats.

CHAPTER 2
IMPACT OF VOLUNTARY EXERCISE ON RENAL NITRIC OXIDE AND ANTIOXIDANT STATUS: A STRAIN DIFFERENCE COMPARISON

Background

One mechanism by which the vasculature benefits from exercise is by increases in blood flow. This increases shear stress which induces the nitric oxide (NO) generating enzyme, endothelial NO synthase (eNOS), and the antioxidant, extracellular superoxide dismutase (EC SOD) (Fukai *et al.* 2000; Harrison *et al.* 2006; Davis *et al.* 2003). As a result, endothelium-dependent vasodilation improves and the risk for developing cardiovascular disease is reduced (DeSouza *et al.* 2000).

In the kidney, however, renal blood flow (RBF) falls during exercise (McAllister, 1998; Tidgren *et al.* 1991; Mueller *et al.* 1998; Musch *et al.* 2004) and this could lead to falls in NO and antioxidant bioavailability (Miyauchi *et al.* 2006; Middlekauff *et al.* 1997). The impact of chronic exercise on the intrarenal NO and antioxidant systems are poorly understood. This is clinically relevant since chronic kidney disease (CKD) is associated with a high level of cardiovascular risk (Hostetter, 2004; Anavekar & Pfeffer, 2004), and exercise is often prescribed to combat cardiovascular-related complications. Since NO deficiency contributes to CKD progression (Baylis, 2009), any intervention that impairs renal NO production could accelerate the underlying kidney disease.

Therefore, the main objective of this study was to investigate the impact of chronic voluntary exercise on the renal cortical abundance of eNOS and EC SOD. In order to determine whether the duration of training influences the renal endothelial responses we compared 6 and 12 weeks of voluntary exercise. We also conducted the study in two different rats strains with differing cardiovascular risk profiles; the Sprague Dawley rat which is susceptible to development of experimentally-induced, as well as age-

dependent hypertension (Erdely *et al.* 2003), and the Fisher 344 (F344) rat which is resistant (Hall *et al.* 1976; Goldstein, 1988). In addition to renal eNOS and EC SOD, we also determined the abundance of other anti- and pro-oxidant proteins, the eNOS localization using immunohistochemistry and also measured indices of oxidative stress, NO production and renal function.

Methods

Animal Procedures

All aspects of animal handling were approved and in accordance with the University of Florida's Institutional Animal Care and Use Committee. Male Sprague Dawley (38) and Fisher 344 (31) rats at 10-12 weeks of age were purchased from Harlan (Indianapolis, IN). Rats from each strain, SD and F344, were either maintained as sedentary (SED) controls or were given 24 hour *ad libitum* access to voluntary wheel running (VWR; Lafayette Instruments, Lafayette, IN) exercise (EX). Running wheels were attached to odometers that calculated distance run and data was acquired using the Activity Wheel Monitor Software (Lafayette Instruments, Lafayette, IN). All rats were singly housed in a temperature and light-controlled environment with access to standard rat chow and water.

The following groups were studied: In the 1st series of studies SD rats were run for 3 weeks (n=3) and compared to controls (n=3) or 6 weeks (n=7) and compared to controls (n=5). The 6 week VWR and SED rats had been implanted with telemetry probes ~10 days prior to randomization into groups and mean blood pressure (BP) and heart rate (HR) were measured at baseline and then once/week for 6 weeks with continual recording over a 24h period. Rats were removed from running wheels and

sacrificed under isoflurane anesthesia within 30 min and the soleus muscle and kidneys were harvested, and the cortex separated and flash-frozen in liquid nitrogen.

In the 2nd series, SD (n=3) and F344 (n=3) were allowed access to VWR for 6 weeks and were compared to SED SD (n=5) and SED F344 (n=4), respectively. At the end of the 6 week period and to allow determination of total NO production (from urinary NO_2 + NO_3 = NO_x) all rats were placed on a nutritionally complete, low nitrate (AIN-76C, MP Biomedicals, Solon, OH) diet for 24 hours, and then housed in metabolic cages for overnight collection of urine. The following day rats were anesthetized with isoflurane and sacrificed which in the case of the EX rats meant ~24h after cessation of VWR. The abdomen was opened and the aortic bifurcation cannulated, a blood sample withdrawn, and then the kidneys perfused with cold PBS and the left kidney was removed and the cortex flash-frozen in liquid nitrogen. The perfusate was then switched to a 2% paraformaldehyde-lysine-periodate (PLP) and the right kidney perfused for 5 minutes. A slice of the perfused kidney was placed in the same fixative for 24 hours at 4ºC, and then transferred into cold PBS where it remained at 4ºC for further analyses (see below).

In the 3rd series SD (n=6) and F344 (n=12) rats were allowed 12 weeks access to VWR and compared to SED SD (n=6) and SED F344 (n=12), respectively. In these rats the right kidney cortex was removed and flash frozen in liquid nitrogen for later analysis.

Immunohistochemistry

PLP-perfused kidneys were embedded in polyester wax, sectioned at a thickness of 5 µm, and then mounted onto glass slides pre-treated with gelatin. Sections were dewaxed, peroxidase blocked for 45 minutes then washed with distilled water. To reduce background, sections were steamed in antigen retrieval solution (DAKO) for 30

minutes, cooled for 20 minutes, and then blocked with protein blocker (DAKO) for 15 minutes. Next, sections were placed in a humidified tray and incubated overnight with the mouse monoclonal eNOS antibody (BD Transduction; 1:5000) at 4ºC. The following day sections were washed with PBS, incubated for 30 minutes with one drop of MACH2 mouse HRP-polymer secondary antibody (Biocare Medical), washed again with PBS, and then incubated with diaminobenzidine for 5 minutes. Sections were then dehydrated with xylene, mounted onto cover slips using Eukitt mounting medium (Sigma), and allowed to dry flat prior to observation by light microscopy.

Preparation for Telemetry

In a preliminary operation, under isoflurane anesthesia and using full sterile technique, a catheter was fed under the skin by trocar and introduced into the left femoral artery. The catheter was tied into position, and the C40 transmitter unit was sutured to the internal abdominal wall. Rats were singly housed and allowed to recover for ~10 days. Blood pressure (BP) and heart rate (HR) were measured using the DSI equipment and software (St Paul, MN).

Western Blot

The relative protein abundance of eNOS (BD Transduction; 1:250), SOD isoforms (Stressgen Reagents; EC SOD 1:250, CuZn SOD 1:2000, and Mn SOD 1:2000), p22phox (Santa Cruz Biotechnology, Inc.; 1:50), and nitrotyrosine (Millipore; 1:500) in renal cortical tissue were measured by Western Blot as previously described (Moningka *et al.* 2011). Briefly, 200 µg of homogenized kidney cortex were separated by electrophoresis (7.5% or 12% acrylamide gel, 140 V, 65 min), and then transferred onto nitrocellulose membranes (GE Healthcare) for 60 min at 0.18 A. Membranes were stained with Ponceau Red (Sigma) to check for transfer efficiency/uniformity and equal

loading, incubated in blocking solution for 60 min, and washed in TBS + 0.05% Tween before overnight primary antibody incubation at 4°C. Membranes were then incubated with the appropriate secondary antibody for one hour, washed and developed with enhanced chemiluminescent reagents (Thermo Scientific). Bands were quantified by densitometry using the VersaDoc™ Imaging System and One Analysis Software (BioRad). Protein abundance was calculated as integrated optical density (IOD) of the protein of interest (after subtraction of background), factored for Ponceau Red stain (total protein loaded) and positive control, then expressed as a % change from the respective SED control.

Analytical Methods

As previously described, plasma and urine creatinine concentrations were measured by HPLC, and urine protein levels were detected using the Bradford method (Sasser *et al.* 2009). Citrate synthase activities in soleus tissue in units of uM/min/g wet tissue weight were based on methods adapted by Srere (Srere, 1969).

Statistical Analyses

Data are presented as mean ± SE and analyzed with the unpaired Student *t-test* between SED and VWR of each strain using GraphPad Prism software (San Diego, CA) Significance was defined as $p<0.05$.

Results

Running activity in both strains is given in Table 2-1 for the series 3 experiments where rats were exposed to 12 weeks of VWR. Running increased gradually over the first 3 weeks of exposure to VWR and by week 4 a maximum value was reached that remained steady thereafter (Table 2-1). The majority of activity occurred during the wake (data not shown) cycle in both strains. As shown in Fig. 2-1, BW increased with

age in all rats but in both strains the rate of rise was attenuated with VWR when compared to SED rats.

In the 1st series of rats, running activity showed a similar gradual increase as compared to SD rats of the 3rd series in Table 2-1 (from 1.1±0.3, to 2.1±0.4, to 3.3±0.7 km/day at weeks 1, 2, and 3). This level of activity was not sufficient to produce an increase in soleus muscle citrate synthase activity (20.1±3.4 vs. 21.9±2.7 μM/min/g wet weight in SED and 3 weeks VWR, respectively), and as shown in Fig. 2-2*A*, there was no difference in the kidney cortex abundance of either eNOS or EC SOD in SED vs. EX. In the rats allowed 6 weeks of VWR, running activity had reached a plateau of ~4.7±1.6 km/day after week 4 and there was a clear elevation in soleus muscle citrate synthase activity (19.1±1.2 vs. 24.3±0.6 μM/min/g wet weight in SED and EX, respectively; $p<0.05$). We also observed that HR fell significantly as a result 6 weeks VWR (348±5 and 319±9 bpm, SED and EX, respectively; $p<0.05$), although BP was not altered (105±5 vs. 97±9 mmHg, SED and EX, respectively). As shown in Fig. 2-2*B* both eNOS and EC SOD abundance in kidney cortex were markedly reduced after 6 weeks of VWR.

In the 2nd series we compared SD and F344 rats exposed to 6 weeks VWR vs. SED. Both running activity and BW changes followed the same patterns shown in Table 2-1 and Fig. 2-1(data not shown). In the SD rats both eNOS and EC SOD were again reduced in EX vs. SED (Fig. 2-2*C*) although the magnitude of the fall in eNOS was blunted compared to the 1st series 6 week VWR rats (Fig. 2-2*B*); perhaps reflecting the 24h break from exercise prior to sacrifice. In contrast, in the F344 rat, 6 weeks VWR led to increased renal cortex eNOS while EC SOD was unchanged with EX (Fig.

2-2*C*). For both rat strains, eNOS localized predominately to the endothelial lining of vessels of the kidney (Fig. 2-3) and 6 weeks of VWR did not change eNOS localization in either strain. However, eNOS staining decreased in intensity in the SD with VWR (Fig. 2-3*A*-*B*), whereas in the F344 rat, eNOS staining increased (Fig. 2-3*C*-*D*), in accordance with the Western blot data. No significant differences in PCr, CCr, and UpV values were detected between SED and 6 wk VWR groups within each strain (Table 2-2).

In the 3rd series 12 weeks of VWR again significantly decreased kidney eNOS and EC SOD in SD in contrast to the F344 rat where marked increases in eNOS and EC SOD occurred (Fig. 2-2*D*). In SD, 12 weeks of VWR had no effect on renal CuZn SOD but increased Mn SOD, while abundance of both enzymes was increased with VWR in F344 (Fig. 2-4). Interestingly, absolute values for renal eNOS in the SED SD rat were significantly greater than the SED F344 rat (5.7±1.10 vs. 2.3±0.30 IOD/Ponceau/Positive Control, SD vs. F344, respectively; $p<0.05$). Moreover, absolute values for renal EC SOD in the SED SD rat were significantly lower compared to the SED F344 rat (10.2±1.92 vs. 16.3±0.72 IOD/Ponceau/Positive Control, SD vs. F344 respectively; $p<0.05$). Strain differences were detected in the renal oxidative stress response to exercise (Fig. 2-5); 12 weeks VWR decreased p22phox abundance and had no effect on H_2O_2 or nitrotyrosine levels in the SD rat. In contrast, the F344 rat exhibited increases in both kidney cortex p22phox and H_2O_2 while nitrotyrosine was unchanged with 12 weeks of VWR.

Discussion

The main novel finding in this study is that the impact of chronic exercise on eNOS and EC SOD in the kidney is variable and influenced by genetic background. Despite

comparable running profiles and equivalent renal functional responses to 6-12 weeks of VWR, we detected profound differences in the kidney cortex's response to exercise between the young adult male SD and F344 rat. In the SD rat, VWR significantly *decreased* kidney cortex eNOS, EC SOD and p22phox whereas in the F344, VWR *increased* these variables. Immunohistochemical studies confirm that the strain dependent changes in eNOS occur exclusively in the vascular endothelium. These directionally opposite changes in eNOS and EC SOD abundance between the two rat strains suggest that while chronic mild exercise may have beneficial renal vascular effects in the F344, it could be damaging to the SD.

Shear stress is the frictional force of blood against a vessel wall and is considered a primary signal for exercise-induced endothelial adaptations. With increases in blood flow and therefore increases in shear stress, the endothelium responds by producing NO, an essential vasodilator that has anti-atherogenic properties (Harrison *et al.* 2006). In addition, endothelial NO directly up-regulates the production of the antioxidant EC SOD (Fukai *et al.* 2000). The importance of shear stress in mediating exercise-induced increases in eNOS/EC-SOD is emphasized by the finding that mice deficient in c-Src (a tyrosine kinase required for the shear response), failed to increase aortic eNOS and ECSOD protein levels following exercise (Davis *et al.* 2003). The increased production of vascular EC SOD during exercise is critical to offset one potentially negative effect of exercise, namely increased metabolism leading to increased generation of reactive oxygen species (ROS) in the vasculature. The net result of these increases in vascular eNOS and EC SOD will be an improvement in endothelial function.

Indeed, numerous studies report improved endothelial-dependent vasodilation with exercise training (Green *et al.* 2009; Jasperse *et al.* 2006; Laughlin, 1995). However, in the kidney, blood flow *falls* with exercise. The exercise-induced renal vasoconstriction occurs rapidly and probably involves increased renal sympathetic nerve activity as well as activation of various vasoconstrictor agents (Mueller *et al.* 2004; *Stebbins et al.* 1995, Ahlborg *et al.* 1995; Maeda *et al.* 2004; Stebbins *et al.* 1993). The degree of renal vasoconstriction is exercise intensity dependent, for example, in male SD rats, renal blood flow falls ~ 50% during mild exercise, ~75% with moderate and <90% with severe treadmill running (McAllister, 1998). During moderate exercise, glomerular filtration rate (GFR) is well maintained despite falls in RBF, probably due to increased glomerular blood pressure. As exercise intensity increases and further reductions in renal blood flow occur, GFR also falls. Transient, exercise-induced proteinuria occurs, possibly secondary to increased glomerular BP (Poortmans, 1988). Of note, both glomerular hypertension and increased protein excretion can lead to kidney damage (Brenner *et al.* 2006; Abbate *et al*. 2006). In addition, when renal vasoconstriction occurs during exercise, shear stress may be reduced within parts of the renal vasculature, leading to reduction in local eNOS and ECSOD. Therefore, all of these “normal” renal responses to exercise could have adverse effects on the kidney. As discussed in a recent article by Padilla *et al.,* exercise-induced endothelial adaptations in vascular beds not involved in the hyperemic response warrant further study (Padilla *et al*. 2011).

In the Wistar rat, Miyauchi and colleagues reported decreased renal eNOS mRNA, protein, and enzyme activity in previously exercise-trained rats subjected to 45 min at 25m/min of acute treadmill exercise, whereas in the lung, where blood flow increases

with exercise, these variables increased (Miyauchi *et al.* 2003). Our observations in the SD extend these findings and show that mild chronic exercise in the SD led to a sustained decrease in eNOS and EC SOD in kidneys harvested ~30 min after 6 weeks VWR. This effect persisted 24 hours after exercise after both 6 and 12 weeks, and apparently required a "training effect" since with only 3 weeks VWR, there was neither an increase in soleus muscle citrate synthase nor any change in renal eNOS and ECSOD. We chose to use the low-intensity VWR exercise protocol since it does not require the use of air jet stress or electric shock to motivate animals to run. A limitation of VWR is that running activity is variable among rats since total distance and intensity of exercise cannot be controlled. In this study, despite variation in running activity in rats, the majority of running occurred during the rat's dark or 'wake' cycle and the distance run was similar in both strains.

Despite the similarity in VWR activity between the rat strains, there were profound differences in the renal responses since in the F344 rat, exercise *increased* eNOS and EC SOD abundance. These are surprising differences in the renal response to exercise between normal young adult males of the two strains. Since the strain difference is seen with VWR it is unlikely to reflect different stress responses, which could be a concern for forced exercise. Both SD and F344 exhibit intensity dependent falls in RBF with treadmill running (McAllister, 1998) although there has been no direct comparison between the two strains. If the F344 have a more efficient cardiac output response to low intensity exercise vs. SD they may not exhibit a fall in RBF with low intensity VWR but this remains to be determined. Alternatively, both strains may undergo renal vasoconstriction and falls in RBF with VWR but the pattern of intrarenal shear stress

may vary, since shear stress will depend on flow but also on vessel radius and local viscosity. The renal circulation is very complex with intricate branching patterns (Moffat & Fourman, 1963) and there may be architectural differences within the renal vasculature of the two strains that create different local shear responses. These are intriguing possibilities that merit further study since genetic background may also determine the renal eNOS and EC SOD responses to exercise in man. If so, knowledge of the renal exercise phenotype would be important in determining recommended exercise intensity and modality. It is certainly true that at high exercise intensities, acute kidney injury due to rhabdomyolysis and/or dehydration can develop in normal individuals (Clarkson, 2007; Seedat *et al.* 1990; Yan *et al.* 2010; Bosch *et al.* 2009). It may be that exercise-induced loss of renal eNOS and EC SOD predisposes to acute kidney injury.

In addition to the strain dependent differences in renal EC SOD in response to exercise, we also observed that while renal Mn SOD increased with exercise in both strains, CuZn SOD abundance increased only in F344. Further, abundance of renal p22phox (NADPH oxidase subunit) fell with exercise in SD but rose in F344. These findings suggest that in the SD reductions in eNOS and EC SOD were counterbalanced by antioxidant effects (increased MnSOD and falls in p22phox). In the F344 where p22phox increased, there was also an increase in the CuZn SOD and in both strains the unchanged renal nitrotyrosine level suggested no net alteration in renal oxidative stress.

In conclusion, this study provides evidence of a strain difference in the renal response to voluntary exercise. The loss of eNOS and EC SOD seen in the SD rat could render the kidney vulnerable to superimposed AKI. The cause for the strain

difference is currently unknown but may be related to different intrarenal hemodynamic responses to exercise.

Table 2-1. Voluntary wheel running activity presented as average daily km run per day.

	Sprague Dawley (n=6)	Fisher 344 (n=12)
Week 1	0.89±0.31	1.92±0.32
Week 2	3.60±1.19	2.79±0.52
Week 3	4.03±1.27	3.16±0.48
Week 4	4.79±1.67	3.33±0.53
Week 6	4.23±1.57	2.42±0.34
Week 9	4.05±1.68	3.37±0.54
Week 12	2.05±1.11	2.59±0.34

Table 2-2. Renal functional responses in SD and F344 after 6 weeks voluntary exercise

	SD		F344	
	SED (n=6)	EX (n=3)	SED (n=4)	EX (n=3)
PCr (mg/dl)	0.10±0.01	0.08±0.00	0.08±0.01	0.07±0.01
CCr (mL/min/100g BW)	2.47±0.24	2.97±0.14	2.69±0.35	3.07±0.27
UpV (mg/day/100g BW)	8.15±1.35	5.99±1.47	6.09±0.14	6.88±0.22

SD, Sprague Dawley; F344, Fisher 344; SED, sedentary; EX, exercise; PCr, plasma creatinine; CCr, creatinine clearance; UpV, urinary protein excretion. *$p<0.05$ vs. respective SED; +$p<0.05$ vs. SED EX.

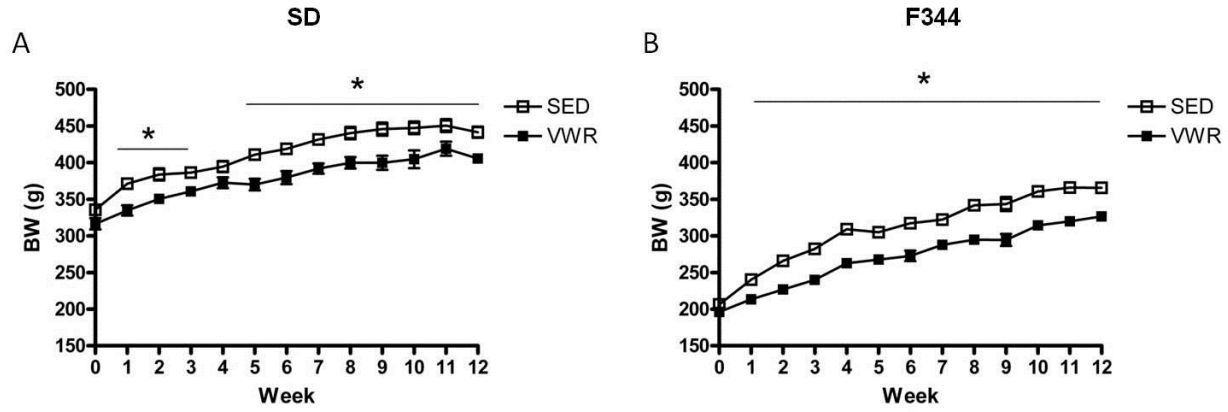

Figure 2-1. Body weight (BW) measurements in the (A) Sprague Dawley (SD) and (B) Fisher 344 (F344) rat. Voluntary exercise effectively reduced BW throughout the 12 week training period in both the SD and F344 rat.

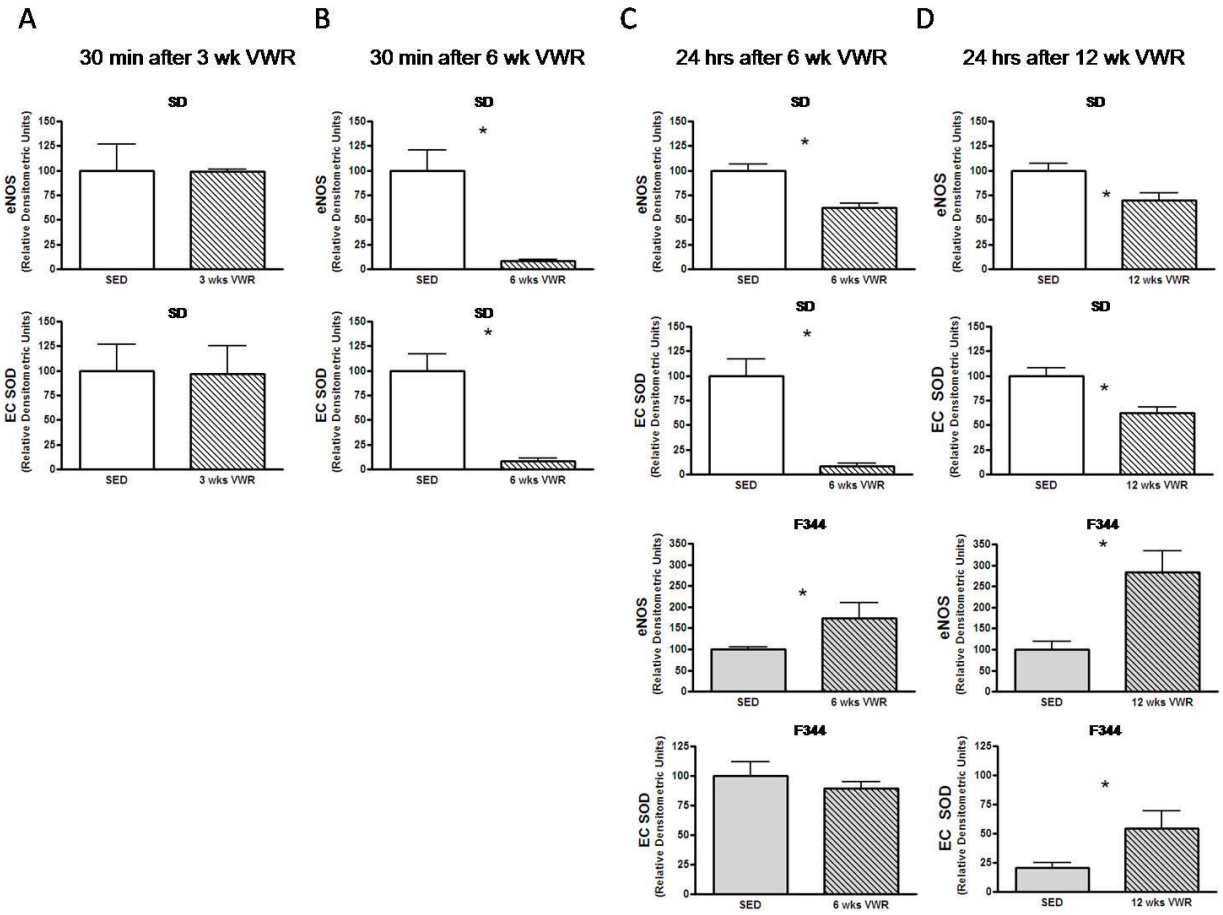

Figure 2-2. Kidney cortex endothelial nitric oxide synthase (eNOS) and extracellular superoxide dismutase (EC SOD) abundance after 3, 6, or 12 weeks of voluntary wheel running (VWR) in the Sprague Dawley (SD) and Fisher (F344) rat. In the SD rat, no changes in eNOS or EC SOD were detected after (A) 3 weeks of VWR, whereas significant falls were observed after (B & C) 6 and (D) 12 weeks of VWR. In the F344 rat, both (C) 6 and (D) 12 weeks of VWR increased eNOS and EC SOD. Relative density units were expressed as a % from respective SED controls. *Denotes a statistical significance of $p<0.05$ between the two groups.

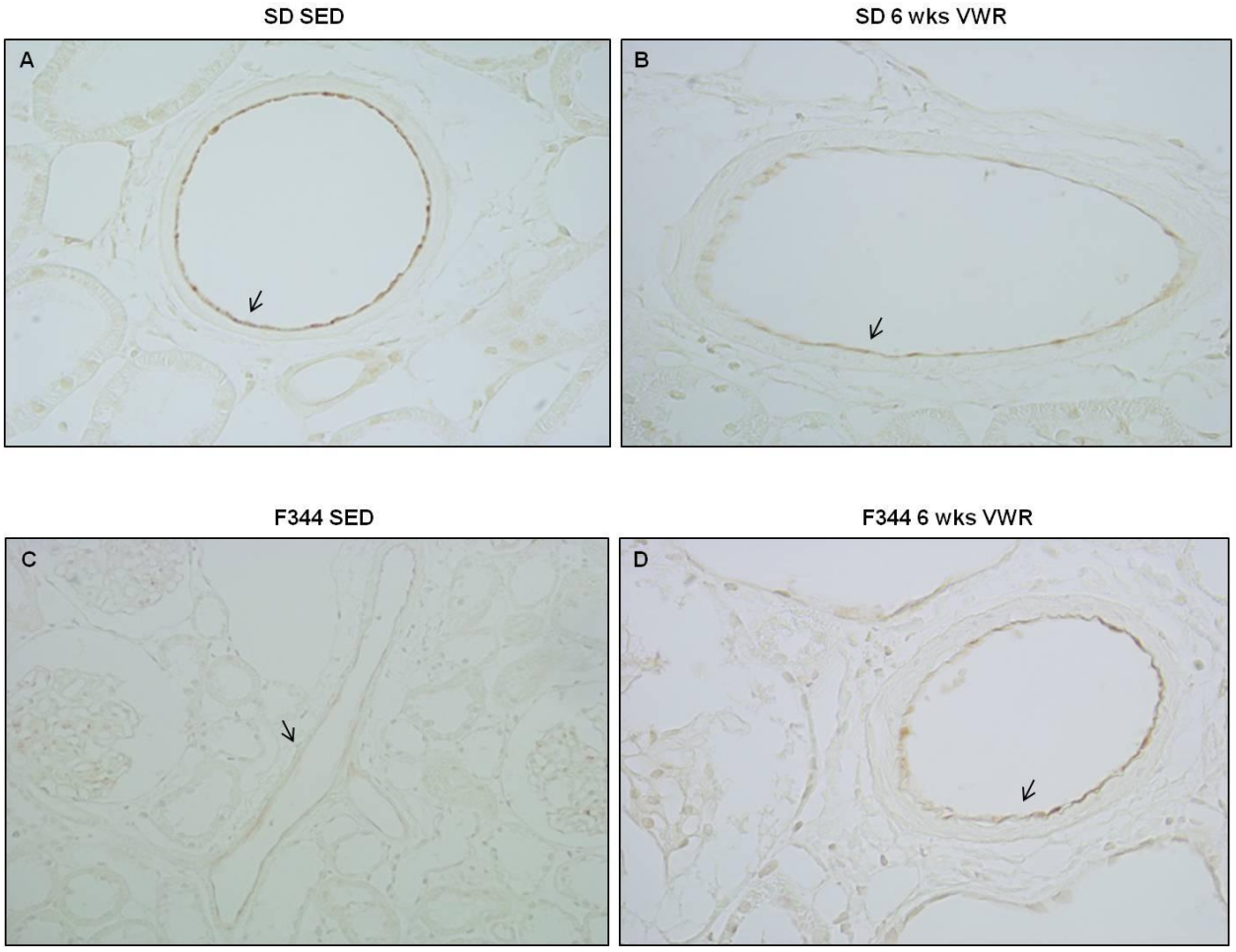

Figure 2-3. Effect of 6 weeks voluntary wheel running on the immunoreactivity of endothelial nitric oxide synthase (eNOS) in the kidney of (A,B) Sprague Dawley and (C,D) Fisher 344 rats. For all groups, eNOS predominately localizes to the endothelium of vessel walls (indicated by arrowheads). Localization was unchanged after 6 weeks of VWR in either strain. However, in the SD, eNOS immunoreactivity decreased with exercise, whereas in the F344, it increased.

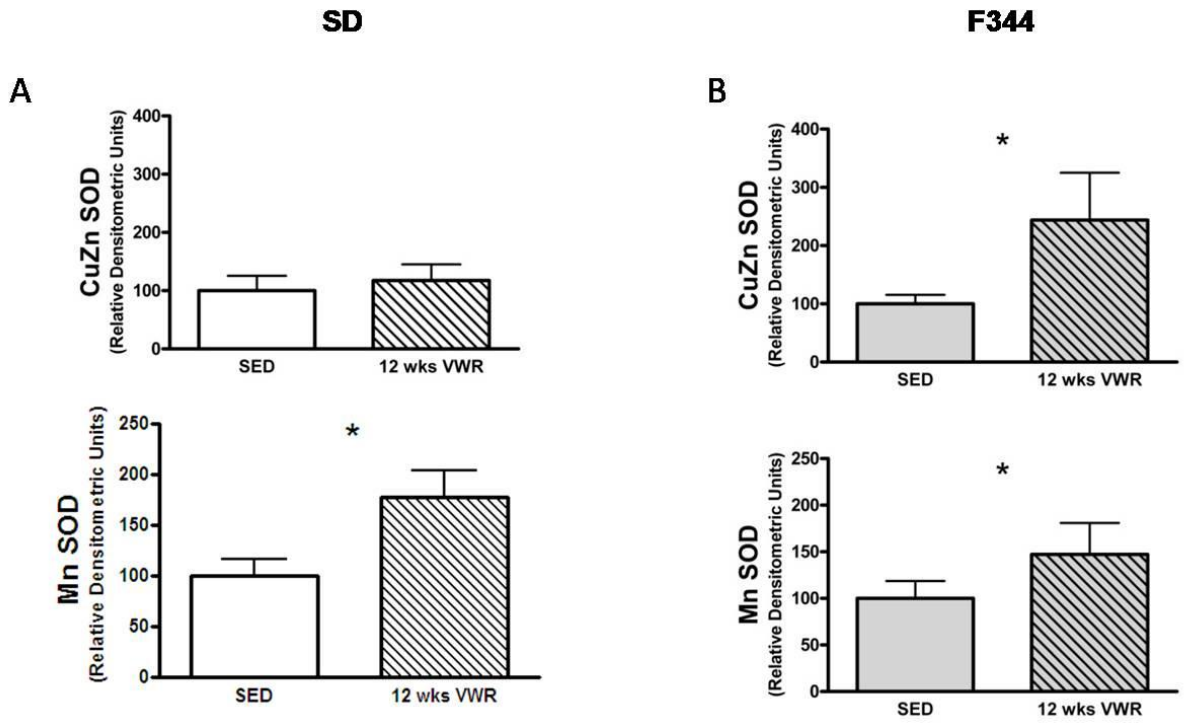

Figure 2-4. Impact of 12 weeks voluntary wheel running (VWR) on CuZn superoxide dismutase (CuZn SOD) and manganese SOD (Mn SOD) in the kidney cortex of the (A) Sprague Dawley (SD) and (B) Fisher 344 (F344) rat. 12 weeks of VWR increased CuZn SOD in the F344 only and increased Mn SOD for both strains. Relative density units were expressed as a % from respective SED controls. *Denotes a statistical significance of $p<0.05$ between the two groups.

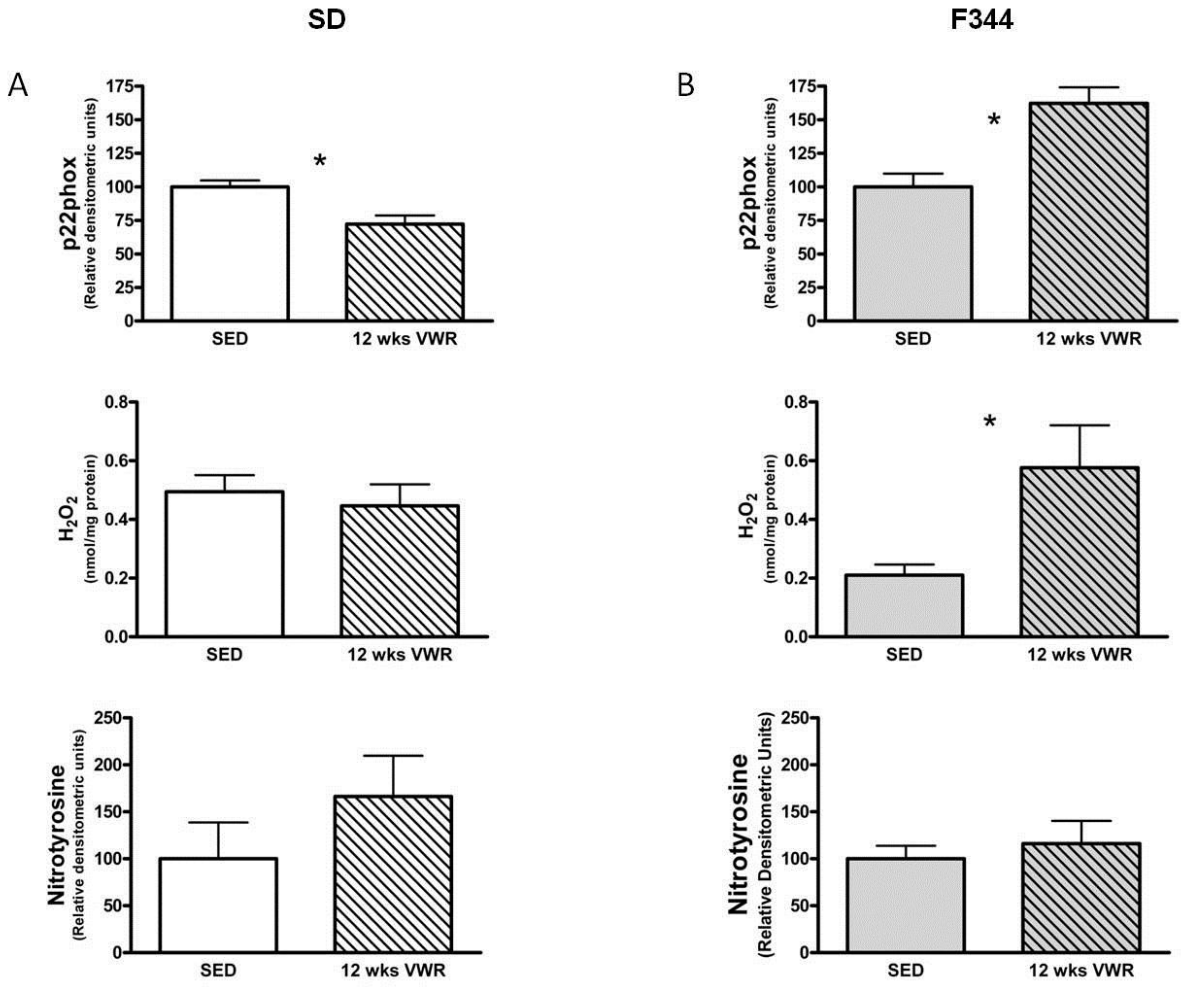

Figure 2-5. Impact of 12 weeks voluntary wheel running (VWR) on oxidative stress; p22phox protein and nitrotyrosine protein in the kidney cortex of the (A) Sprague Dawley (SD) and (B) Fisher 344 (F344) rat. In the SD rat, 12 weeks VWR decreased p22phox, whereas in the F344 rat it increased. No differences were detected in nitrotyrosine abundance in either strain. Relative density units were expressed as a % from respective sedentary (SED) controls. *Denotes a statistical significance of $p<0.05$ between the two groups.

CHAPTER 3
EXERCISE EXACERBATES ISCHEMIA-REPERFUSION INDUCED ACUTE KIDNEY INJURY IN THE SPRAGUE DAWLEY BUT NOT FISHER 344 RAT

Background

In vessels of skeletal muscle, lung, and heart, exercise increases blood flow which imparts increased shear stress on the endothelium leading to increases in vasodilatory molecules such as nitric oxide (NO). In addition to increased NO, exercise also stimulates expression of the antioxidant, extracellular superoxide dismutase (EC SOD) (Fukai *et al.* 2000). Applied in the disease setting, exercise significantly improves impaired endothelial-dependent vasodilation associated with aging (Spier *et al.* 2007), diabetes (Sakamoto *et al.* 1998), and hypertension (Higashi *et al.* 1999). Thus, one mechanism by which exercise is beneficial is through the induction of endothelial eNOS and EC SOD in the vasculature.

Despite increasing evidence for the beneficial role of NO in exercise hyperemia, its role in tissues where blood flow reduces is less understood. For instance, in the kidney, blood flow is markedly decreased, a process dependent on intensity and increased sympathetic outflow (Poortmans 1990; Tidgren *et al.* 1991; Mueller *et al.* 1998). With an acute bout of high intensity treadmill exercise, Miyauchi et al. reported significant decreases in renal endothelial NO synthase (eNOS) mRNA, protein, and enzyme activity. Although not measurable the authors concluded that exercise decreased shear stress in the kidney leading to falls in eNOS. Furthermore, exercise-trained mice deficient in c-Src, a critical component of the shear stress signaling cascade, failed to increase aortic eNOS and EC SOD protein compared to exercise-trained wild-type mice (Davis *et al*. 2002). Altogether, these studies suggest the importance of blood flow in dictating increased NO production and EC SOD with exercise.

As previously reported in Chapter 2, we found that in the Sprague Dawley (SD) rat VWR EX leads to falls in renal eNOS and EC SOD which may render the kidney vulnerable to ischemia/reperfusion (IR) induced acute kidney injury (AKI), an injury associated with reduced NO production and increased oxidative stress (Noiri *et al.* 2001). Thus, the aim of the present study was to investigate if exercise exacerbates IR-induced AKI in the SD. Strain differences were also explored since predisposition to chronic kidney disease (CKD) varies between the Sprague Dawley and Fisher 344, with the latter being more protected (Erdely *et al.* 2003; Moningka *et al.* 2011). Based on our finding reported in Chapter 2 that in the F344 VWR EX results in *increased* renal eNOS and ECSOD, we hypothesized that the exercised F344 might be protected against IR-induced AKI. Finally, in an effort to define a hemodynamic mechanism for the different renal eNOS/EC SOD responses to VWR exercise training in the two strains, we also employed the radiolabeled microsphere method to determine renal blood flow during exercise. Because of technical issues we used mild treadmill exercise (TM EX) rather than VWR EX in these studies.

Methods

Animal Procedures

All animal handling was in accordance with and approved by the University of Florida's Institutional Animal Care and Use Committee. Male Sprague Dawley (SD) and Fisher 34 (F344) rats 10-12 weeks of age were purchased from Harlan (Indianapolis, IN). All rats were singly housed in a temperature and light-controlled environment with access to standard rat chow and water.

For each strain, rats were randomly divided into a sedentary control (SED; SD n=12; F344 n=15) or voluntary exercise (VWR EX; SD n=13; F344 n=15) group. For 12

weeks SED rats led a sedentary lifestyle, whereas the VWR EX rats were given individual running wheels with 24 hour ad libitum access (Lafayette Instruments). VWR activity was measured by attached odometers and acquired using the Activity Wheel Monitor Software (Lafayette Instruments, Lafayette, IN). At the end of the 12 weeks, rats were prepared for right uninephrectomy (UNX) and ischemia/reperfusion (IR)-induced acute kidney injury (AKI) to the left kidney, ~24 hours after running cessation.

All surgeries were with full sterile technique. While under general anesthesia using isoflurane (5% induction and 1-2% maintenance dose), the left renal pedicle was clamped for 35 minutes. In order to compare injury susceptibility between SED and VWR EX groups, we chose a clamping period that did not cause maximal injury, and based on preliminary studies 35 minutes met these criteria. To maintain tissue moisture, 0.9% sterile saline solution was applied over the ischemic kidney. During the 35 minute ischemic period, we performed UNX of the right (normal) kidney. Cortical sections of the right kidney were immediately flash frozen in liquid nitrogen and stored at -80ºC for further analyses. Incisions were closed with Vicryl silk (internal suture) and stapled (external suture). The analgesic buprenorphine (0.05 mg/kg body weight) was injected subcutaneously and the rat recovered for the next 24 hours. This allowed the kidney to reperfuse 24 hours prior to a renal function study. Additional studies were performed in F344 SED (n=9) and F344 VWR EX (n=9) and SD SED (n=10) and SD VWR EX (n=7) with right UNX only.

All rats were then prepared for terminal inulin clearance studies 24 hours after UNX-IR or UNX surgery for determination of glomerular filtration rate (GFR) and renal plasma flow (RPF). Rats were anesthetized with Inactin (intraperitoneal injection, 120

mg/kg BW) and placed on a heating table to maintain a body temperature of 37±1°C. The trachea was cannulated with PE-240 tubing and exposed to a constant flow of oxygen. Using PE-50 tubing filled with heparinized saline, the femoral artery was cannulated for measurement of blood pressure (BP) and for collection of blood samples. A baseline BP was taken as well as a blood sample of ~250 µL for measurement of creatinine. The femoral vein was then cannulated and a 0.5 mL bolus of pre-dialyzed FITC-Inulin (final concentration 2 mg/mL 0.9% NaCl; Sigma) was infused; thereafter, FITC-inulin was infused at a rate of 1.2 mL/100g BW/hr. To ensure that the rat was euvolemic artificial plasma containing 5% bovine serum albumin/γ-globulin was infused at a rate of 1% 100g BW/hr for the first fifteen minutes, and then at a rate of 0.15% 100g BW/hour for the remainder of the study. Next, the abdominal cavity was opened to expose the left kidney and bladder. A non-occluding catheter was placed in the left renal vein to obtain an arterial-venous inulin extraction for calculation of RPF. The bladder was cannulated with flanged PE-50 tubing for collection of urine and for gravemetric determination of urine volume and flow rate. The level of anesthesia, BP, and body temperature were monitored throughout. After a 60 minute stabilization period, two x 20 minute urine collections with mid-point collections of femoral arterial and renal venous blood were taken for FITC-Inulin measurement. All blood samples were centrifuged for collection of plasma which was analysed for FITC inulin content and together with urine samples were used to calculated .glomerular filtration rate (GFR, from inulin clearance), renal plasma flow (RPF, from (Urine inulin concentration / plasma A-V inulin extraction)/urine flow) and filtration fraction (FF, from GFR/RPF). The rat was sacrificed by exsanguination and the lung and left kidney (with IR-induced AKI)

were removed. A portion of the kidney was prepared for histology (see below), and the remaining divided into sections of cortex. Lung and kidney tissue were flash frozen in liquid nitrogen and stored at -80ºC for further analyses.

Renal Pathology

Histology-related procedures were performed in the Gainesville Veterans Affairs Medical Center's Pathology Clinical Laboratory. Kidney tissues were fixed in 10% formalin (Sigma) for 48 hours, processed, embedded in paraffin wax, and then cut into 5 µm sections. All sections were stained with periodic acid schiff (Sigma) and counterstained with hematoxylin and eosin (Sigma). B.P. Croker, an expert renal pathologist, scored all tissue sections for acute tubular necrosis while blinded by the treatment groups. He evaluated the tubules of the renal cortex for evidence of cell swelling, brush border loss, nuclear condensation, karyolysis (dissolution of the nucleus), regeneration, capillaritis (endothelial inflammation), and cell sloughing. Each category was given a score as follows: a grade of "0" signified that none of the tubules were involved, 1 = ≤10%, 2=11 – 25%, 3=26 – 50%, 4=51 – 75%, 5=76 –100% were involved.

Western Blot

The protein abundance of endothelial nitric oxide synthase (eNOS), extracellular superoxide dismutase (EC SOD), cytosolic copper zinc SOD (CuZn SOD), mitochondrial manganese SOD (Mn SOD), and p22phox were detected in whole kidney cortex tissue homogenates as previously described (Moningka *et al.* 2011). Whole lung tissue were homogenized similarly and detected for eNOS and EC SOD abundance only. Kidney cortex and lung samples were run on 7 or 12% acrylamide gels (BioRad) and then semi-dry transferred (BioRad) onto nitrocellulose membranes (Biorad). To

confirm equal loading, membranes were stained with Ponceau Red (Sigma), a dye that stains for protein. Primary antibody concentrations were as follows: 1) eNOS (BD Transduction; 1:250), 2) EC SOD (Abcam; 1:250), 3) CuZn SOD (Stressgen; 1:2000), 4) Mn SOD (Stressgen; 1:2000), and 5) p22phox (Santa Cruz; 1:50). Membranes were developed with chemiluminescent reagents and bands at the expected molecular weight were analyzed by densitometry using the VersaDoc™ Imaging System and One Analysis Software (BioRad).

Analytical Methods

FITC-Inulin concentrations (mg/mL) in urine and plasma were measured by fluorescence (excitation and emission wavelengths, 485 nm and 530 nm, respectively) as previously described (Knight *et al.* 2007). Creatinine concentrations (mg/dl) in plasma were measured by HPLC (Sasser *et al.* 2009).

Blood Flow Measurements

Blood flow measurements to the kidney, mesentery, and select muscles of the hindlimb were conducted on a separate set of male SD and F344 rats (10-12 weeks of age; SD n=13; F344 n=9) using the reference sample microsphere method (Laughlin *et al.* 1982; Behnke *et al.* 2006). A subset of sedentary F344 rats were provided from a previous study (Dominguez *et al.* 2010) for at rest RBF measurements (unpublished) only (n=10). For these studies, training and acute exercise was by TM EX since injection of microspheres through indwelling catheters would interfere with the voluntary wheel apparatus. Initially, all rats were habituated on a motor-driven rodent treadmill. During this period, rats walked/ran at an initial intensity of 5 m/min for 5 min/day and at zero degree incline for 5 days. Intensity progressed to 15 m/min by day 5. Rats were then randomly separated into SED (SD; n=7 and F344; n=3) and TM EX (SD; n=6 and

F344; n=6) groups. SED rats remained sedentary for 10-12 weeks while TM EX rats were gradually conditioned to run at an intensity of 15 m/min, 5 days/week, and 15º incline for 20 minutes at week 1, 30 minutes at week 2, 40-50 minutes at week 3, 50-60 minutes at week 4, and then 60 minutes at week 5. TM EX rats continued to run for 60 minutes at the same intensity and incline for the remainder of the study. This training protocol effectively increases skeletal muscle citrate synthase activity (Delp *et al* 1993). Training adherence was prompted with small bursts of compressed air and/or a low-voltage electric grid at the back of the treadmill; however, these tactics were used mainly during the first three weeks and minimally thereafter.

At the end of the 10-12 weeks, all rats were surgically implanted with indwelling catheters in their carotid and caudal arteries as previously described using PE-10 and PE-50 tubing, respectively (Behnke *et al.* 2006). All surgeries were performed with sterile technique and while the rat was under general isoflurane anesthesia. Both catheters were exteriorized through the skin, primed with heparinized saline, and then plugged. After incisions were closed, rats were given the analgesic bupivacaine (0.5 mg/mL), and then returned to individual clean cages where they were carefully monitored during a recovery period of ≥ 2 hours.

Next, all rats, including SED, were placed on a motor-driven treadmill. Running speed gradually increased to 15 m/min (15º incline) over 30 s and then maintained. While running, the rat's caudal artery catheter was attached to a heparinized-saline syringe connected to a Harvard infusion-withdrawal pump set at a withdrawal rate of 0.25 mL/min. After 3 min of treadmill running, radiolabelled microspheres (^{46}Sc or ^{85}Sr, randomly chosen; PerkinElmer NEN™) were injected into the carotid artery.

Microspheres were sonicated and vortexed prior to injection to prevent clumping and to ensure thorough mixing. The carotid artery catheter was then flushed with 0.5 mL of warm (37ºC) 0.9% saline, and connected to a pressure transducer for measurement of BP. Withdrawal of the reference blood sample started ~30 s prior to microsphere infusion and continued for ~30-60 s after injection to ensure that all microspheres were cleared from the catheter line. Exercise was then terminated.

The rat was then allowed to rest for 30 min after which a second microsphere injection (using the other radioactive label) was performed in a similar approach as just described, except that the microsphere injection and withdrawal of the reference sample were conducted while the animal rested quietly. To ensure optimal resting conditions, room lights were dimmed while the rat situated itself in a corner of the treadmill that was topped with a cover. Rats were then euthanized with an overdose of sodium pentobarbital (>100 mg/kg) injected into the carotid artery. The kidneys, gastrocnemius, and mesentery were carefully dissected, weighed, and placed into tubes. For the gastrocnemius muscle, the red portion was dissected. All blood and tissue samples were detected for their level of radioactivity with a gamma counter (Packard Cobra II Auto-Gamma). Individual tissue blood flows were calculated using the reference sample method (Ishise *et al*. 1980). Adequate microsphere distribution was confirmed by demonstrating a <31% difference in blood flow between paired kidneys.

Statistical Analyses

All data are presented as mean ± SE and analyzed with the unpaired or paired Student *t-test,* or with ANOVA using GraphPad Prism software (San Diego, CA). Significance was defined as $p<0.05$.

Results

Body weight (BW) responses to VWR EX and running activity were comparable in both strains (Fig. 3-1). In each respective strain, baseline BW's were similar between SED and VWR EX. Despite gradual increases in BW in both SED and VWR EX, presumably due to normal rate of growth, VWR EX significantly reduced BW compared to SED in both strains. Running activity steadily increased from weeks 1 to 4, reaching a plateau of ~3-4 km/day, a pattern demonstrated for both strains, although the SD rat demonstrated greater variability.

As illustrated in Table 3-1, the renal hemodynamic response to 24h UNX in the SD rat was similar between SED and VWR EX groups, showing falls in GFR and RPF by ~ 50% of control (~1 mL/100g BW/min). When IR was superimposed on UNX much greater falls in GFR and RPF occurred in both SED and EX groups and plasma creatinine (PCr) rose markedly. However, the fall in GFR and rise in PCr were greatly exacerbated in the VWR EX SD rats subjected to UNX-IR compared to SED, due to greater falls in RPF and FF in the EX group (Table 3-1).

In the F344 rat, as seen in the SD rat, the renal hemodynamic response to UNX was similar in SED and VWR EX groups (Table 3-2). Again combination UNX and IR lead to increases in PCr and falls in GFR, RPF, and FF compared to UNX alone. In contrast to SD, there was no difference in the severity of the falls in GFR and RPF and increase in PCr in the EX vs. SED F344.

The combination of UNX and IR led to structural changes in kidney cortex characteristic of AKI which in the SD tended to be worse in the VWR EX rats and there was significant brush border loss compared to SED (Fig. 3-2*A*). In the F344 rat, UNX-

IR generally affected SED and VWR EX groups to the same degree, although there was less cell sloughing in the VWR EX rats (Fig. 3-2*B*).

VWR EX reduced kidney cortex eNOS (by 16%), EC SOD (by 38%), and p22phox (by 28%) abundance in the normal (right) kidney of the SD rat (Fig. 3-3*A*). IR injury significantly increased eNOS abundance similarly for both SED and VWR EX groups, but had no further effect on EC SOD and p22phox abundance in the SD rat. By contrast, in the F344 rat, VWR EX increased eNOS (by >100%), EC SOD (by >100%), and p22phox (by 62%) kidney cortex abundance in the normal kidney (Fig. 3-3*B*) and IR injury markedly increased eNOS in a similar manner for both SED and VWR EX groups. In F344 rats, EX reduced EC SOD with IR; however, no further changes were detected in p22phox abundance with IR injury in either group of F344 rats. In the lung, where blood flow increases with exercise, the abundance of both eNOS and EC SOD increased significantly with VWR EX (Fig. 3-4).

For determination of RBF during TM EX, additional rats of each strain comparable in age were obtained, also from Harlan (Indianapolis, IN). In the SD rat, 10-12 weeks of TM EX reduced BW, increased the soleus weight: BW ratio and had no effect on left ventricle (LV):BW ratio (Fig. 3-5*A*). In the F344 rat, 10-12 weeks of TM EX increased LV:BW but had no effect on BW or soleus weight (Fig. 3-5*B*).

As shown in Fig. 3-6*A*, blood flow to the red portion of the gastrocnemius increased in SED SD whereas blood flow to the kidney and mesentery decreased during a ~4 minute bout of TM EX (15 m/min). The chronically TM EX-trained SD rat showed similar trends but only mesenteric blood flow fell significantly with acute exercise (Fig. 3-6*A*). During the bout of TM EX, SED F344 rats increased red

gastrocnemius blood flow and decreased blood flow to the kidney while the decline in mesenteric blood flow was not significant. Whereas the increase in gastrocnemius blood flow persisted with acute exercise in the TM EX trained rats, the fall in RBF was completely abolished (Fig. 3-6*B*). Of note, the resting RBF (factored for BW) was remarkably similar in both SED SD and SED F344 (597±48 and 592±51µL/min) and also in VWR EX-trained SD and F344 rats (332±106 and 373±72 µL/min). In both strains chronic exercise training lowered the resting value of RBF by ~35-45% (both $p<0.05$).

Discussion

The main novel findings in this study are that 1) whereas chronic VWR EX causes directionally opposite changes in renal cortical eNOS and EC SOD in SD vs. F344, in the lung, increases in both enzymes are seen in both strains, 2) 12 weeks of VWR EX exacerbates IR-induced falls in GFR in the young adult male SD but not in the F344 rat, 3) using the radiolabeled microsphere technique, we found that TM EX leads to significant and similar reductions in resting RBF in both rat strains. In the untrained SED animals, acute exercise significantly and similarly reduces RBF in both strains while with training the acute RBF response to TM EX is blunted in SD and is absent in the F344.

There is substantial evidence provides benefit in skeletal muscle and coronary blood vessels and protects the heart against IR-induced injury (Sindler *et al.* 2009; Fogarty *et al.* 2004; Powers *et al.* 2007). Several mechanisms are involved in the cardiac protection including the up-regulation of myocardial antioxidant capacity which helps combat the increased content of reactive oxygen species, lipid peroxides, protein oxidation, and protein nitration associated with myocardial IR (Powers *et al.* 2007). The

exercise-induced increases in antioxidant capacity are through increased shear stress which stimulates NO production and in turn, also stimulates production of the EC SOD antioxidant. This was confirmed by a study that discovered a lack of EC SOD up-regulation with exercise in aorta (where shear stress increases) in eNOS deficient mice (Fukai *et al.* 2000). Pulmonary blood flow (and shear stress) increases with exercise and as shown here eNOS and EC SOD abundance in the lung increase with exercise in both SD and F344. However, in the kidney, blood flow is thought to be reduced during exercise. The eNOS mRNA, protein abundance, and enzyme activity decreased with an acute bout of treadmill exercise in trained Wistar rats, suggesting that shear stress is reduced in the kidney (Miyauchi *et al.* 2000). Exhaustive exercise leading to functional and structural kidney damage in the untrained SD rat also decreased renal cortical NOS and SOD activity (Lin *et al.* 2010). In Chapter 2, we reported that chronic VWR EX reduced renal eNOS and EC SOD in the SD rat, whereas in the F344 rat, these beneficial enzymes increased. We confirmed this response of increased eNOS and EC SOD in the F344 rat using chronic TM EX (Moningka *et al.* 2011, in revision).

Falls in NO bioavailability can render the kidney susceptible to injury since NO is critical for normal renal function and its deficiency leads to chronic kidney disease, CKD (Baylis 2007). In addition, endothelial injury is a major mechanism by which IR induced AKI propagates. There is recruitment of local inflammatory signals which up-regulate reactive oxygen species leading to reductions in NO bioavailability and therefore development of renal dysfunction (Le Dorze *et al.* 2009). Several studies have reported cases of renal ischemia leading to exercise-induced AKI (Seedat *et al.* 1990; Yan *et al.* 2010; Bosch *et al.* 2009) some of which is due to rhabdomyolysis which leads to

myoglobin-induced AKI (Bosch *et al.* 2009). Also, when there is pre-existing oxidative stress, development of exercise-induced AKI is high, for example, in patients with renal hypouricemia (Yan *et al.* 2010; Saito *et al.* 2011; Ishikawa 2002). Based on these findings that exercise may cause/exacerbate AKI and on our observed strain differences in renal eNOS and EC SOD with exercise training, we conducted the present study to determine whether exercise influences susceptibility to UNX-IR induced AKI in the two strains.

Despite comparable running activities and BW responses to VWR training, we discovered differences in the impact of VWR EX on susceptibility to IR injury between the SD and F344 rat. In the SD rat, 12 weeks of VWR EX reduced renal eNOS and EC SOD, rendering the kidney susceptible to IR-induced AKI since VWR EX exacerbated the falls in GFR, RPF, and increased PCr associated with UNX-IR. In contrast, we found that in the F344 rat, 12 weeks of VWR EX *increased* renal eNOS and EC SOD abundance and that the chronically exercised F344 showed some protection against the decline in renal function with IR. We also discovered a strain difference in susceptibility to IR in SED rats with the F344 more vulnerable. UNX-IR reduced GFR by 63% and 87% in the SD and F344 rat, respectively. Moreover, there were also greater reductions in RPF with UNX-IR in the F344 rat versus SD rat (65% vs. 78%, SD vs. F344, respectively). Despite greater reductions in renal function to UNX-IR, exercise afforded protection in the F344 rat. Overall, our data suggest that vulnerability to an oxidative-stress mediated renal insult such as IR-induced AKI is determined by the state of endothelial health which is influenced by genetic background.

It is interesting that UNX-IR had no effect on either kidney cortex EC SOD or p22phox in either strain. Moreover, the directional responses of EC SOD, and p22phox to VWR EX were also unchanged by IR, again for both strains. These data indicate that despite the decline in renal function with UNX-IR seen for both strains, the antioxidant/ pro-oxidant EC SOD and p22phox were not influenced by IR injury. We also found that IR *increased* eNOS abundance in both the SED and VWR EX rats of both strains. NOS protein has been shown to increase with IR injury but this is usually the inducible form of NOS associated with inflammation (Goligorsky *et al.* 2002; Chatterjee *et al.* 2002); the inducible NOS isoform was not investigated in the present study. While the increased eNOS with IR may represent an attempt at compensation, falls in renal function and histological evidence of injury occur in both strains. Despite a lack of marked differences in each index, we found that in the IR-susceptible SD rat, brush border loss was greater in the VWR EX IR compared to the SED EX IR which is in accordance with the greater functional injury with UNX-IR seen in the exercised SD.

Despite potential injury associated with renal ischemia, a reduction in RBF due to exercise is considered a normal physiological response since it allows diversion of blood to accommodate the increase in oxygen demand by active muscles (McAllister 1998). Evidence points to increased renal sympathetic nervous system outflow as the primary mechanism (Tidgren *et al*. 1991). Using exercise-trained rabbits, Mueller et al. reported that dose-dependent increases and concomitant falls in renal blood flow caused by exercise were partly due to activation of the α-adrenergic receptors (Mueller *et al.* 1998). Angiotensin II, endothelin-1, and vasopressin are also involved (Stebbins *et al.* 1995, Ahlborg *et al.* 1995; Maeda *et al.* 2004; Stebbins *et al.* 1993). Despite falls

in renal blood flow, glomerular filtration rate is well-maintained at low intensities due to compensatory increases in filtration fraction; however, at high intensities, renal blood flow is severely compromised (Poortmans & Vanderstraeten, 1994).

To determine whether there were differences in renal hemodyamic responses to exercise between the two strains, which might account for the differences in renal endothelial enzymes with exercise, we utilized the radiolabeled microsphere method to measure real time total RBF during exercise and at rest. We found that in the SD rat, blood flow during exercise increased in the red gastrocnemius but decreased in the kidney and mesentery; however, with chronic TM EX, the magnitude of fall in RBF during exercise decreased. We also found that in the F344 SED rat, kidney blood flow significantly reduced during acute exercise but that this response was lost in the TM EX group. These findings agree with observations by Armstrong and Laughlin who showed that in SD rats chronic exercise reduced the magnitude of the decrease in RBF after acute treadmill-exercise (Armstrong & Laughlin 1984).

In conclusion, this study provides evidence that genetic background influences susceptibility to IR-induced AKI. While running activity and BW responses to VWR were comparable between the male SD and F344 rat, their functional responses to UNX-IR were different. While UNX-IR injury markedly reduced renal function in both SED and EX of both strains, in the SD, VWR EX *exacerbated* falls in GFR, RPF, and PCr. This is in contrast to the F344 rat, where VWR EX provided some protection against renal UNX-IR injury. Indices of acute structural injury in the kidney cortex also support these functional data. These findings align with our observations that 12 weeks of VWR EX reduced eNOS and EC SOD renal cortical abundance in the SD rat but increased eNOS

and EC SOD renal cortical abundance in the F344 rat. However, these strain differences are not related to different renal hemodynamic responses to chronic and acute exercise since resting RBF fell similarly with exercise training in both strains, and the robust response to acute exercise seen in SED rats was blunted by exercise-training in both strains. Perhaps differences in intrarenal vascular architecture and shear stress patterns can account for the different eNOS and EC SOD responses to exercise seen in the two rat strains.

Table 3-1. Sprague Dawley – Renal hemodynamic responses

	SED		VWR EX	
	UNX	UNX-IR	UNX	UNX-IR
GFR (mL/min/100g BW)	0.47±0.08	0.17±0.04*	0.45±0.03	0.04±0.01*+
RPF (mL/min/100g BW)	2.74±0.82	0.96±0.23*	1.83±0.26	0.30±0.15*+
FF	0.24±0.02	0.19±0.03	0.28±0.03	0.12±0.02*
PCr (mg/mL)	0.28±0.02	1.07±0.27*	0.27±0.03	2.42±0.35*+

GFR, glomerular filtration rate; RPF, renal plasma flow; FF, filtration fraction; PCr, plasma creatinine. *p<0.05 vs. respective UNX, +p<0.05 vs. SED UNX-IR.

Table 3-2. Fisher 344 – Renal hemodynamic responses.

	SED		VWR EX	
	UNX	UNX-IR	UNX	UNX-IR
GFR (mL/min/100g BW)	0.47±0.03	0.06±0.01*	0.58±0.04	0.16±0.06*
RPF (mL/min/100g BW)	1.84±0.23	0.40±0.10*	2.53±0.26	1.02±0.35*
FF	0.28±0.02	0.20±0.02*	0.25±0.02	0.21±0.05
PCr (mg/mL)	0.25±0.04	1.77±0.19*	0.29±0.02	1.78±0.28*

GFR, glomerular filtration rate; RPF, renal plasma flow; FF, filtration fraction; PCr, plasma creatinine. *p<0.05 vs. respective UNX.

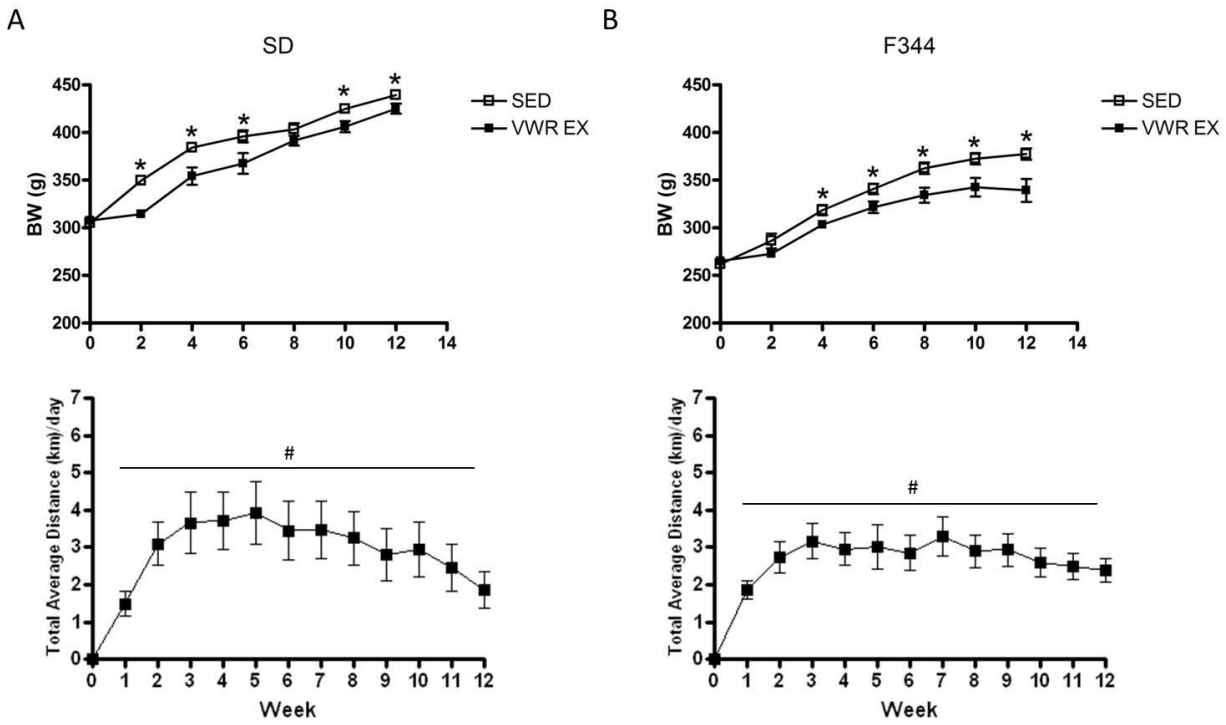

Figure 3-1. Effect of 12 weeks voluntary exercise (VWR EX) on body weight (BW) and running activity in the (A) Sprague Dawley and (B) Fisher 344 rat. Voluntary exercise effectively reduced BW throughout the 12 week training period in both the SD and F344 rat. For each strain, total average distance (km) run per day steadily increased over the first few weeks and remained relatively constant for the remainder of the study. *Denotes a statistical significance of $p<0.05$ between the two groups. #Denotes a statistical significance of $p<0.05$ compared to baseline.

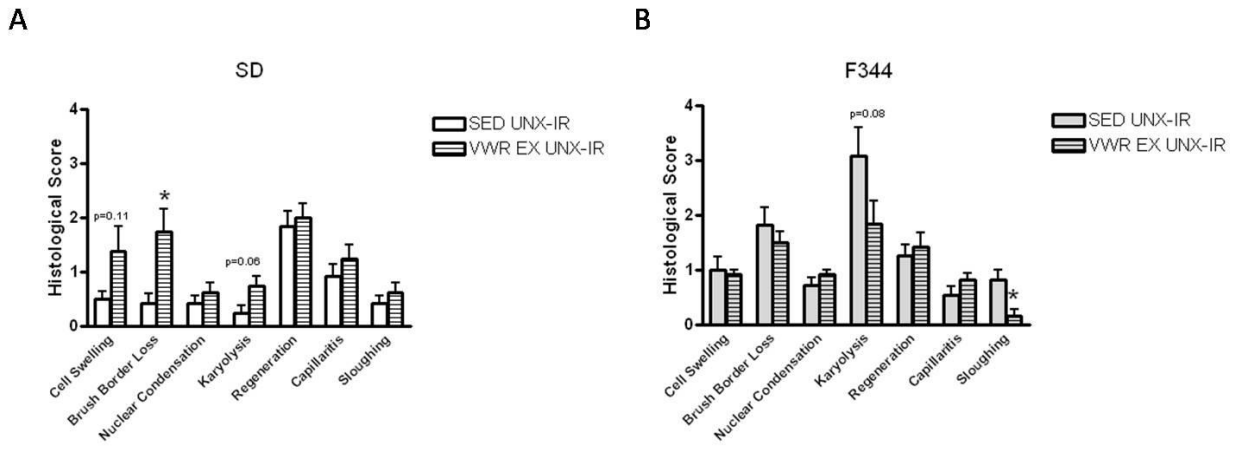

Figure 3-2. Effect of 12 weeks voluntary exercise (VWR EX) and renal uninephrectomy (UNX) ischemia/reperfusion (IR) injury on indices of acute renal structural injury in kidney cortex of (A) Sprague Dawley (SD) and (B) Fisher 344 (F344) rats. Analyses were conducted on IR kidneys only since minimal to no injury was expected for UNX alone. In the SD rat, EX and IR tended to increase all markers of acute injury and significantly increased the level of brush border loss. In the F344 rat, IR affected SED and EX groups to the same degree; however, 12 weeks of VWR EX ameliorated the level of sloughing against IR. *Denotes a statistical significance of $p<0.05$ compared to SED IR.

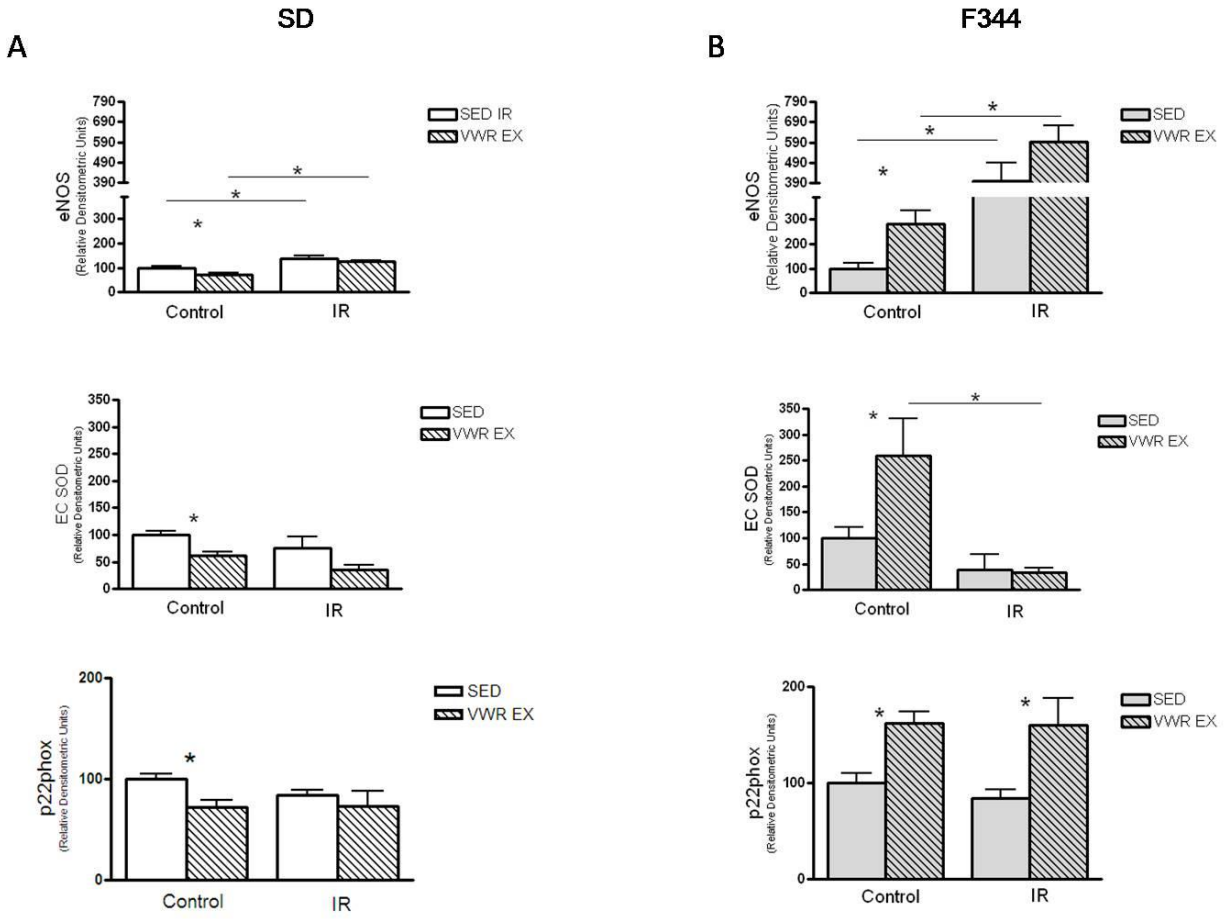

Figure 3-3. Effect of 12 weeks voluntary exercise (VWR EX) and renal uninephrectomy (UNX) and ischemia/reperfusion (IR) injury on endothelial nitric oxide synthase (eNOS), extracellular superoxide dismutase (EC SOD), and p22phox abundance in kidney cortex of (A) Sprague Dawley (SD) and (B) Fisher 344 (F344) rats. In the SD rat, VWR EX reduced eNOS, EC SOD, and p22phox, whereas in the F344 rat, these proteins increased. For both strains, IR increased eNOS for both SED and VWR EX groups but had no further effect on EC SOD and p22phox abundance. *Denotes a statistical significance of $p<0.05$ between the two groups.

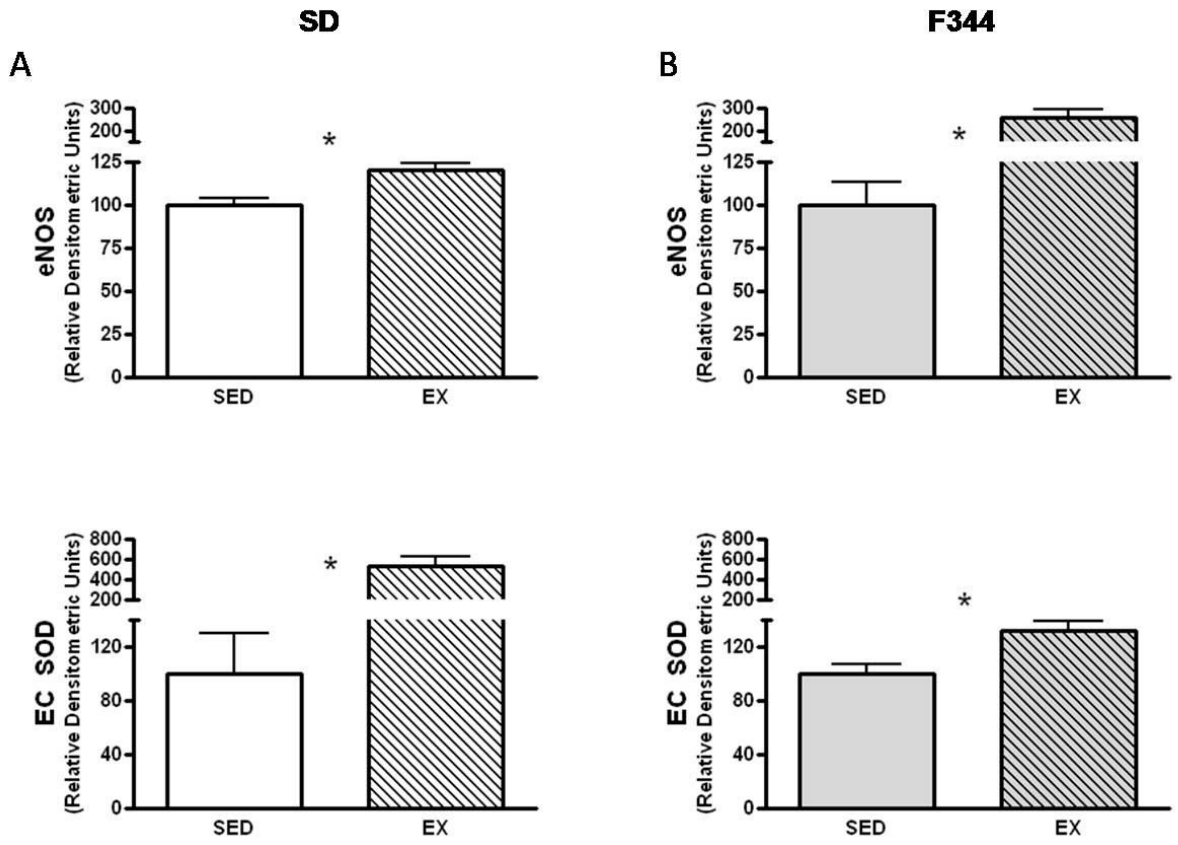

Figure 3-4. Effect of 12 weeks voluntary exercise (VWR EX) and renal uninephrectomy (UNX) and ischemia/reperfusion (IR) injury on endothelial nitric oxide synthase (eNOS), extracellular superoxide dismutase (EC SOD), and p22phox abundance in lung of (A) Sprague Dawley (SD) and (B) Fisher 344 (F344) rats. For both strains, VWR EX significantly increased eNOS and EC SOD in lung. *Denotes a statistical significance of $p<0.05$ between the two groups.

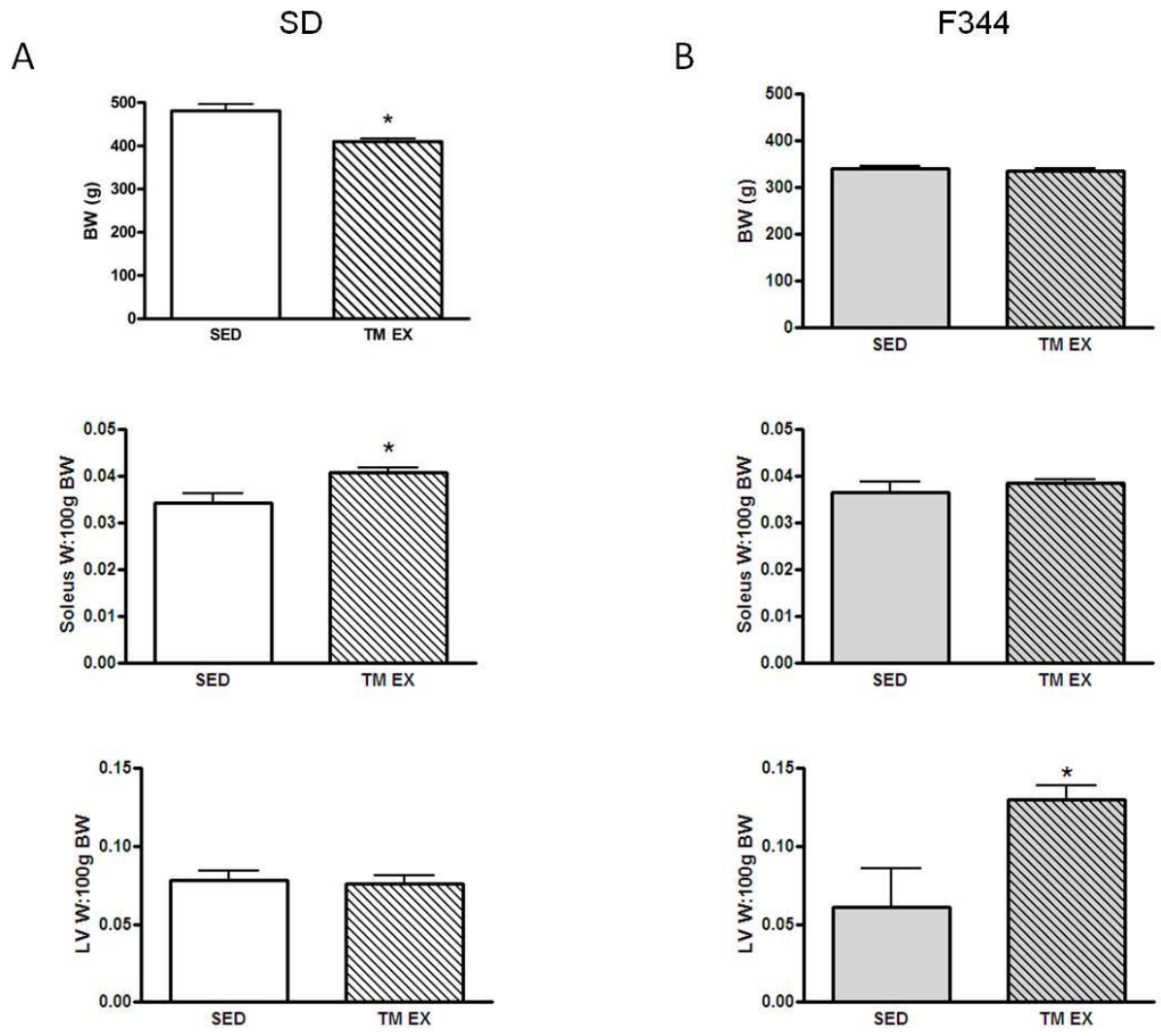

Figure 3-5. Effect of 10-12 weeks treadmill exercise (TM EX) on final BW, soleus weight:BW, and left ventricular (LV) weight:BW in the (A) Sprague Dawley (SD) and (B) Fisher 344 (F344) rat. In the SD rat, TM EX significantly reduced BW, increased soleus weight but had no effect on LV weight. In the F344 rat, despite no effect of TM on BW and soleus weight, LV hypertrophy was evident. *Denotes a statistical significance of $p<0.05$ between the two groups.

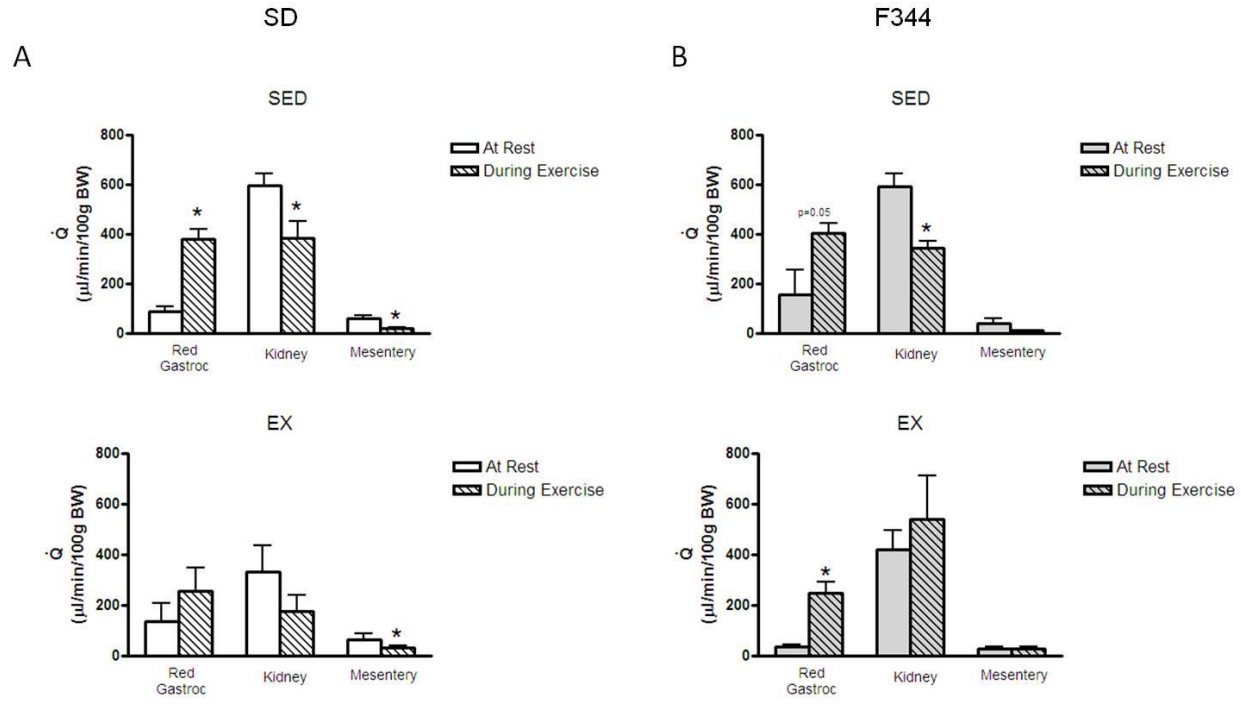

Figure 3-6. Blood flow (Q) measurements in sedentary (SED) and treadmill exercise (TM EX) rats chronically trained for 10-12 weeks taken at rest and during exercise in the (A) Sprague Dawley (SD) and (B) Fisher 344 (F344) rat. In the SD SED rat, blood flow during exercise increased in the red gastrocnemius (gastroc) but decreased in the kidney and mesentery; however, with chronic TM EX, the changes in red gastrocnemius and kidney blood flow were blunted while mesentery blood flow reduced during exercise. In the F344 SED rat, only kidney blood flow significantly reduced during exercise, a response that was lost in the TM EX group. Further, red gastrocnemius blood flow significantly increased during exercise in the chronically TM EX-trained F344 rat. *Denotes a statistical significance of $p<0.05$ compared to rest. Note: For red gastroc and mesentery BF in SED F344 n=3 but for RBF in SED F344 n=13; see methods for explanation.

CHAPTER 4
TWELVE WEEKS OF TREADMILL EXERCISE DOES NOT REVERSE AGE-DEPENDENT CHRONIC KIDNEY DISEASE IN THE FISHER 344 MALE RAT

Background

Exercise reduces morbidity and mortality from various cardiovascular diseases in the elderly population (Larson & Bruce, 1987). It has beneficial metabolic actions including reduction in plasma triglycerides, increases in the high-density lipoprotein to low-density lipoprotein ratio and insulin sensitivity, and improves cardiac function (Heath 1983; Sun *et al*. 2008). Physical activity may also reduce depression and mental stress thus indirectly improving blood pressure (Paluska & Schewnk, 2000).

Another beneficial response to exercise is the stimulation of endothelial nitric oxide synthase (eNOS), the main enzyme responsible for vascular NO production which is essential for optimal vascular health. An increase in endothelial shear stress as a result of increased blood flow results in 1) prolonged eNOS mRNA stability, 2) increased eNOS protein translation, and 3) increased NOS enzyme activity (Harrison *et al*. 2006). In addition, increased shear stress increases the antioxidant extracellular superoxide dismutase (EC SOD) enzyme by an NO-dependent mechanism (Fukai *et al*. 2000). Mice deficient in c-Src, a critical component of the shear stress signaling pathway, do not increase their aortic eNOS or EC SOD protein abundance with exercise (Davis *et al*. 2003). The shear stress-induced up-regulation of eNOS and EC SOD enhances endothelium-dependent vasodilation in parts of the circulation where blood flow increases during exercise, such as skeletal muscle, pulmonary and coronary circulations (Spier *et al*. 2004; Johnson *et al*. 2000; DeSouza *et al.* 2000; Muller *et al*. 1994). As a result of all these effects exercise has powerful cardiovascular protective actions.

In the kidney, blood flow *decreases* during exercise in an intensity-dependent manner to shunt adequate perfusion to working muscles. At very high exercise intensities renal blood flow can reduce from the normal ~20% of cardiac output to ~1% (Castenfors *et al*. 1967). Under some circumstances a fall in blood flow leads to a reduction in shear stress, which could lead to reductions in endothelial NOS and EC SOD. Indeed, in the male Wistar rat, acute treadmill exercise in pre-trained rats reduced renal eNOS mRNA, protein, and enzyme activity, whereas increases occurred in the lungs where blood flow increases with exercise (Miyauchi *et al*. 2003). Blood flow also falls with exercise in the splanchic circulation and mesenteric arteries isolated from trained rats do not show enhanced flow-mediated dilation in these vascular beds, whereas in skeletal muscle arteries exercise does improve flow-mediated dilation (Sun *et al*. 1998). Thus, one direct endothelial benefit of exercise is shear stress-dependent, and in organs such as the kidney where blood flow falls, depending on how intrarenal shear stress is altered, endothelial function may not be improved, or may even be impaired (Miyauchi *et al*. 2003).

This is of particular concern in the aging kidney where falls in eNOS abundance and NOS activity (Erdely *et al*. 2003) and increased oxidative stress occur (Gomes *et al.* 2009) in conjunction with slowly developing glomerular and tubulointerstitial injury (Baylis & Corman,1998). Superimposing additional eNOS and EC SOD deficits could exacerbate age-dependent kidney damage. Indeed, 6 weeks of exercise in old C57BL/6J mice magnified the age-associated renal structural injury (Lichtig *et al*. 1987).

The main purpose of the present study was to determine the impact of chronic treadmill exercise on the progression of age-dependent renal injury in the male Fisher

344 (F344) rat. We also investigated the effect of exercise on the protein levels of various isoforms of the NOS and SOD enzymes, in kidney cortex and medulla as well as aorta in young and old rats. Furthermore, since reactive oxygen species contributes to the development of age-dependent renal changes, we assessed several markers of oxidative stress (Asghar *et al*. 2007).

Methods

Animal Procedures

All animal handling was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved and monitored by the West Virginia University and University of Florida Institutional Animal Care and Use Committees.

Young (3 months; n=16) and old (22-24 months; n=16) male Fisher 344 (F344) rats supplied from Harlan (Indianapolis, IN) were purchased from the National Institute of Aging (NIA Bethesda, MD). All rats were housed in a temperature/light-controlled environment and given access to standard rat chow and water *ad libitum*. For acclimation purposes, all rats were placed on a motor-driven treadmill for 3 day sessions of 5 min training at an intensity of 5 m/min and zero degree incline. Rats were then randomly assigned to either young or old sedentary (Young SED; Old SED; n=8 for all), or young and old exercise (Young EX; Old EX; n=8 for all) groups. EX rats were trained for 10-12 weeks and SED were age-matched. After a steady increase in treadmill training during the first 3 weeks, EX rats performed 5 days/week for 60 min/day at an intensity of 15 m/min and 15° incline for the remaining weeks. Forty eight hours after their last bout of training, rats were weighed, and anesthetized with isoflurane prior to sacrifice. The kidney, aorta and soleus muscle were dissected and

weighed. A transverse slice of the left kidney was saved in 10% phosphate buffered formalin for histology (see below) and the remaining tissue separated into cortex and medulla. All tissues were flash-frozen in liquid nitrogen, then stored at -80°C until further analyses. For functional measurements, a separate group of young (n=16) and old (n=16) F344 male rats of comparable age, supplied from Taconic Farms (Hudson, NY), were purchased from the NIA. Some rats were treadmill trained (Young EX, n= 10; Old EX, n=8) according to the same protocol described above, while others remained SED (Young SED, n=6; Old SED, n=8). Seven-12 days prior to sacrifice, all rats were placed on a low nitrate, complete diet for 24h (AIN-76C, MP Biomedicals, Solon, OH), then placed in metabolic cages for overnight (~16h) collection of urine. While in the metabolic cages, rats were fasted but were given access to distilled water. Metabolic cage collections did not interfere with the daytime training. Rats were then returned to daily training and regular diet and were sacrificed as described above, and blood was drawn from the aorta, centrifuged, and plasma collected and stored at -80^{o}C. The left kidney was removed and weighed and a section of the kidney was fixed for histology.

Renal Pathology

Fixed kidney tissue was paraffin embedded and 5µm sections were cut and stained with periodic acid schiff (PAS, Sigma, St Louis, MO) followed by hematoxylin as the secondary stain. All glomeruli in the section (>100) were scored, blinded, as follows: 0=healthy glomeruli, +1=<25% damage, +2=25-50% damage, +3=51-74% damage, +4=>75% damage. The glomerulosclerosis index score (GSI) was calculated using the following equation: (#of+1)+ 2(#of+2)+ 3(#of+3)+ 4(#of +4)/ total glomeruli observed.

Western Blot

The proteins measured by Western blot, with their specific primary antibody and concentration given in parenthesis, were: 1) endothelial nitric oxide synthase (eNOS; BD Transduction; 1:250), 2) neuronal (n)NOSα (Santa Cruz; 1:50), 3) nNOSβ (Thermo Scientific, formerly ABR; 1:500), 4) extracellular superoxide dismutase (EC SOD; Abcam; 1:250), 5) cytosolic-copper containing SOD (CuZn SOD; 1:2000), 6) mitochondrial-manganese containing SOD (Mn SOD; 1:2000), 7) p22phox (Santa Cruz; 1:50), and 8) nitrotyrosine (Millipore; 1:500). Homogenized samples of kidney cortex, kidney medulla, and aorta, standardized by protein concentrations (50-200 µg), were separated by electrophoresis (7.5% or 12% acrylamide gel, 200 V, 65 min) and transferred onto nitrocellulose membranes as previously described (Sasser *et al*. 2009). Membranes were stained with Ponceau Red (Sigma) to check for transfer efficiency/uniformity and equal loading, blocked and washed then incubated overnight while on a rocker with primary antibody at 4°C. Membranes were then incubated with the appropriate secondary antibody for one hour at room temperature, and developed with enhanced chemiluminescent reagents (Thermo Scientific). Bands were quantified by densitometry using the VersaDoc™ Imaging System and One Analysis Software (BioRad). Protein abundance was calculated as integrated optical density (IOD) of the protein of interest (after subtraction of background), factored for Ponceau Red stain (a marker of total protein loading) and an internal positive control value (to allow for quantitative comparisons between different membranes). To compare values between kidney cortex and kidney medulla, the protein abundance is represented as

IOD/Ponceau/Control relative to the appropriate control group of the kidney cortex or medulla.

Analytical Methods

As previously described, plasma and urine creatinine concentrations were measured by HPLC, and urine protein levels were detected using the Bradford method (Sasser *et al*. 2009). Hydrogen peroxide (H_2O_2) levels in homogenates of kidney cortex, kidney medulla, and urine were measured using the Amplex® Red Kit (Molecular Probes, Eugene, OR) according to the manufacturer's instructions and signal specificity was confirmed by incubation with 2000 units of catalase (Sigma). Tissue H_2O_2 concentrations (μmol/L) were normalized to total protein concentration (mg/mL) and expressed as nmol/mg protein. Citrate synthase activities in soleus tissue in units of uM/min/g wet tissue weight were based on methods adapted by Srere (Srere P, 1969).

Statistical Analyses

Data are presented as means ± SEM and analyzed using SigmaPlot software (San Jose, CA). The effects of age and exercise training on functional, biochemical and protein abundance data were analyzed by two-way ANOVA, and if found significant, followed by Newman–Keuls post hoc analyses. Renal pathology was analyzed with non-parametric analyses. Significance was defined as $p<0.05$.

Results

The first series of studies on young and old, sedentary and exercise-trained rats were conducted on NIA colony F344 rats obtained from Harlan (Indianapolis, IN). Body weight was higher in old when compared to young rats and exercise reduced body weight in both age groups (Table 4-1). Kidney weight (factored to body weight) revealed hypertrophy with age in the EX group only. As reported previously (Sindler *et*

al. 2009), training efficacy was confirmed with increased activities of citrate synthase in soleus skeletal muscle in both young and old (by ~18% in young rats and by ~20% in old rats). Young rats exhibited minimal glomerular injury and this was not altered by exercise (Fig. 4-1). With age, the index of glomerular sclerosis (GSI; Fig. 4-1*A*) and the %age of total damaged glomeruli (Fig. 4-1*B*), increased substantially and exercise had no effect on renal pathology.

In the kidney cortex, exercise significantly increased eNOS protein abundance in the young rat but had no effect in old (Fig. 4-2*A*). There were no differences in kidney cortex nNOSα abundance due to either age or exercise (Fig. 4-2*B*) and nNOSβ was low and unaffected by EX in young rats while there was a profound increase in nNOSβ abundance in both SED and EX old rats (Fig. 4-2*C*). In the kidney medulla, eNOS protein abundance was higher than in cortex and was similar in old and young rats and not altered by exercise (Fig. 4-2*A*). The abundance of nNOSα in the kidney cortex was unchanged by either exercise or age; however, in the kidney medulla, significance increases were seen with exercise in the old (Fig. 4-2*B*). Age-related increases in nNOSβ protein abundance in both kidney cortex and medulla were unaffected by exercise (Fig. 4-2*C*). The abundance of the nNOSβ in medulla was higher than in cortex in both young groups and was further elevated in the old (Fig. 4-2*C*).

As with eNOS, exercise increased the EC SOD abundance in the kidney cortex of young but not old rats (Fig. 4-3*A*). There were no exercise effects on the other SOD isoforms in kidney cortex or medulla (Fig. 4-3*B* and *C*). With age there were falls in EC SOD and CuZn SOD in both kidney cortex and medulla whereas for Mn SOD, there was no impact of age or exercise in the kidney. Values of EC SOD were higher in medulla

while CuZn and Mn SOD abundance values were lower in medulla vs. cortex in all groups.

The p22phox subunit of the NADPH oxidase increased with age in both kidney cortex and medulla but exercise was without effect (Fig. 4-4*A*). Tissue H_2O_2 levels (Fig. 4-4*B*) and nitrotyrosine abundance (Fig. 4-4*C*) increased with age in both kidney cortex and medulla but were unaffected by exercise. In the aorta of young rats exercise training significantly increased the abundance of eNOS and age increased these levels even further (Fig. 4-5*A*); however, no differences were found between Old SED and Old EX (Fig. 4-5*A*). Aortic EC SOD abundance tended to increased with exercise in the young rats but due to high variability, this did not reach statistical significance (Fig. 4-5B). No further change occurred in aortic EC SOD with age or exercise (Fig. 4-5B). Nitrotyrosine abundance in the aorta significantly rose with age in both groups and EX was again without effect (Fig. 4-5*C*).

For additional functional measurements, later groups of young (n=16) and old (n=16) F344 rats of similar age were obtained from Taconic Farms, also from the NIA colony. Increased BW and renal hypertrophy occurred with age (Table 4-2) as also seen in the Harlan NIA rats (Table 4-1), and the responses to exercise in BW were similar in young and old (Table 4-2). Plasma creatinine (PCr) increased with age in both SED and EX groups, while creatinine clearance (CCr) fell significantly with age in SED only (Table 4-2). Urinary protein excretion markedly increased with age and exercise was without effect in either young or old groups (Table 4-2). To determine total body NO production, we measured the urinary excretion of the stable NO oxidation products, NO_2 and NO_3 (NOx; UNOxV). With age, UNOxV tended to fall; however,

UNOxV significantly increased with exercise in both young and old rats (Table 4-2). No changes in urinary H_2O_2 excretion were detected among any of the groups (3.81±0.56, 5.69±0.99, 4.26±0.80, 2.90±0.75 nmol/24hr/100g BW for Young SED, Young EX, Old SED, and Old EX, respectively). Furthermore, an age-dependent increase in glomerular injury was again observed and this was not improved by exercise (GSI scores: 0.04±0.02, 0.03±0.01, 0.93±0.14^{+}, and 0.78±0.18^{+} for Young SED, Young EX, Old SED, and Old EX, respectively; $^{+}p<0.05$ vs. respective Young).

Discussion

The main finding in the present study was that 10-12 weeks of treadmill exercise increased both eNOS and EC SOD abundance in the kidney cortex of the young rat, despite an expected exercise-induced fall in renal blood flow. There was no change in renal eNOS and ECSOD with exercise. Rats developed age-dependent renal damage and exercise was not able to reverse it. In contrast to other strains, there was also no loss of renal eNOS or nNOS∝ protein in the F344 and abundance of the nNOSβ in the kidney cortex increased >100x due to aging, and exercise did not prevent this. The age-dependent loss of the kidney cortex antioxidants EC SOD and CuZn SOD, and age-dependent increase in p22phox were not influenced by exercise. The second series of rats indicated that with age, F344 rats develop significant proteinuria with some loss of renal function and a tendency for total NO production to fall, and that exercise does not reverse these decremented changes.

The present studies were conducted on the National Institute of Aging's inbred F344 rat, which develops mild-moderate kidney damage with advancing age without systemic hypertension (Wei *et al.* 1986). There are differences in the renal response to aging, with some strains such as the Sprague Dawley being very vulnerable while

others show mild-moderate injury (Munich Wistar and F344), and yet others are resistant (Wag/Rij and F344/ Brown Norway cross) (Baylis & Corman, 1998; Moningka *et al.* 2011). There are also sex differences in the rate of loss of kidney function and development of structural damage in aging man and rats, with the male being most vulnerable (Baylis, 2009; Baylis & Corman, 1998).

We have previously reported that development of severe chronic kidney disease (CKD) is invariably associated with loss of renal cortical nNOS∝ abundance, irrespective of animal model (Baylis, 2008). This includes the aging male Sprague Dawley rat where marked glomerular injury occurs (Erdely *et al.* 2003). The present study demonstrates that the mild-to-moderate renal injury exhibited by the aging F344 rat is not associated with a loss of eNOS or nNOS∝ protein. In contrast, there are large age-dependent increases in the renal cortical abundance of the nNOSβ isoform. We have also observed increased nNOSβ in experimentally-induced forms of CKD, including renal mass reduction (Smith *et al.* 2009; Tain *et al.* 2011) and chronic allograft nephropathy (Tain *et al* 2008). We initially thought that nNOSβ activation in CKD was a secondary, compensatory response to the loss of other NOS isoforms in the damaged kidney. However, we show here that the increased nNOSβ *precedes* loss of the other NOS isoforms, suggesting that this may in fact be involved in *causing* the early, mild-moderate renal damage. There are also antioxidant and pro-oxidant changes in the aging F344 kidney cortex which include loss of both EC SOD and CuZn SOD protein abundance, increased p22phox (NADPH oxidase subunit), increased nitrotyrosine, and increased H_2O_2 levels. These changes may also contribute to the early renal damage.

A main aim of this study was to determine the impact of exercise training on the kidney of the F344 rat. Here, we report that in the young adult F344, exercise increased both eNOS and EC SOD protein in the renal cortex which confirms earlier preliminary findings by us in this strain (Moningka *et al.* 2011). Exercise also increased aortic eNOS indicating that in the F344, these beneficial endothelial enzymes are enhanced by both increased blood flow (to aorta) and decreased blood flow (to kidney). Although not measured by us, rats of various strains including the F344 do reduce their renal blood flow with exercise (Musch *et al*. 2004; Kregel 1995; Laughlin & Armstrong, 1982). Presumably in the young exercise-trained F344 increased intrarenal resistances oppose the decreased flow and lead to increased intrarenal shear stress in some locations, and hence eNOS and EC SOD activation. This is particularly interesting since we previously observed exactly opposite intrarenal effects of exercise in the young male Sprague Dawley rat, where falls in eNOS and EC SOD were seen (Moningka *et al. 2010*). Marked falls in renal blood flow also occur in this strain (Laughlin & Armstrong, 1982). We speculate that in the Sprague Dawley kidney the exercise-induced renal vasoconstriction leads to net falls in intrarenal shear stress. The directional effect of exercise on renal endothelial enzymes has profound consequences since loss of these enzymes renders the Sprague Dawley kidney more susceptible to acute kidney injury, while the F344 strain is protected (Moningka *et al.* 2011). In the present study, however, since the young sedentary F344 has minimal spontaneous injury we did not see any histologic effect of exercise.

Our primary goal was to establish the effect of exercise on renal endothelial enzymes as well as overall renal structure and function in the aging F344 rat. We have

already reported that similar exercise has significant general cardiovascular benefits in the aging F344 leading to enhanced endothelial function in skeletal muscle (Spier *et al.* 2004; Sindler *et al.* 2009). Despite these cardiovascular improvements, we report here that the structural and functional kidney damage in the aging F344 is not reversed by exercise, suggesting that the beneficial effects of the same type/duration of exercise are not inevitably transmitted to the kidney. In fact, there is considerable variability in the reported response of the aging kidney to exercise. In the old (23 months) male Sprague Dawley rat, life-long voluntary wheel running reduced kidney structural damage and was as effective as lifelong caloric restriction (Loupal *et al*. 2005). Of note, in the same study, treadmill running over the same period had no beneficial effects in the Sprague Dawley rat (Loupal *et al*. 2005), similar to our present findings in the old F344 subjected to 10-12 weeks of treadmill running. In contrast, in the old C57BL/6J mouse which develops significant kidney damage, only 6 weeks of forced wheel running considerably *worsened* the age-associated renal structural injury (Lichtig *et al*.1987). The aged F344 rat shows an exaggerated fall in renal blood flow during exercise which we speculate causes unchanged intrarenal shear stress, accounting for the lack of exercise-induced renal eNOS and EC SOD. This lack of activation of these endothelial enzymes, together with loss of CuZn SOD in the aging kidney, which is not restored by exercise, probably contributes to the lack of exercise-induced protection against kidney damage and dysfunction.

Oxidative stress, defined as the imbalance between oxidants and antioxidants, is reported to increase with age, and can be combated with exercise. Moderate exercise reduced age-associated increases in mitochondrial oxidative stress in some organs

(including kidney) of old male mice, although beneficial effects declined in senescent animals (Navarro *et al*. 2004). The same study found that exercise did not prevent the age-dependent decline in kidney CuZn SOD and catalase activity levels (Navarro *et al*. 2004). Here, we report no net effect of exercise on age-related increases in oxidative stress. The protein abundance of p22phox, a subunit of the superoxide generating NADPH oxidase enzyme was up-regulated in both the kidney cortex and kidney medulla with age, and was unchanged with exercise. H_2O_2 levels, an additional marker of oxidative stress, also increased with age in the kidney medulla only, but again, was not affected by exercise. Furthermore, nitrotyrosine, also increased with age in renal cortex, medulla and aorta and was not affected by exercise.

Overall, this study demonstrates that in the young male F344 rat, 12 weeks of treadmill exercise increases kidney cortex eNOS and EC SOD abundance, and that with age, this response is lost. We observed several other age-related changes in the kidney including worsening of renal structural injury, increased renal oxidative stress as detected by increase protein abundance in p22phox, H_2O_2 content and nitrotyrosine, and decreased antioxidant defenses reflected by loss of EC and CuZn SOD. Interestingly, exercise did not prevent any of these adverse changes in the kidney. Therefore, we conclude that 10-12 weeks of chronic treadmill exercise was ineffective in reversing the age-associated declines in renal function and renal antioxidant status in the male F344 rat.

Table 4-1. Characteristics of male F344 rats obtained from Harlan, NIA.

	Young SED	Young EX	Old SED	Old EX
BW (g)	367±8	345±5*	409±9^{+}	377±9*$^{+}$
Kidney wt/100g BW	0.32±0.01	0.30±0.0	0.35±0.02	0.36±0.01^{+}

Sedentary, SED; exercise, EX; body weight, BW. *p<0.05 vs. respective SED. $^{+}$p<0.05 vs. respective Young.

Table 4-2. Characteristics of male F344 rats obtained from Taconic Farms, NIA.

	Young SED	Young EX	Old SED	Old EX
BW (g)	403±12	387±10	456±17^{+}	417±23*
Kidney wt/100g BW	0.26±0.01	0.28±0.01	0.41±0.04^{+}	0.34±0.02
PCr (mg/dl)	0.11±0.02	0.12±0.01	0.22±0.01^{+}	0.20±0.03^{+}
CCr (mL/min/100g BW)	1.98±0.22	1.86±0.12	0.74±0.15^{+}	1.09±0.16
UpV (mg/24hr/100g BW)	2.4±0.2	3.8±0.1	88.8±21^{+}	65.1±17^{+}
UNOxV (umol/24 hr/100g BW)	0.76±0.01	1.21±0.08*	0.46±0.15	0.88±0.10*$^{+}$

Sedentary, SED; exercise, EX; body weight, BW; plasma creatinine, PCr; creatinine clearance, CCr; urinary protein excretion, UpV; urinary nitrate/nitrite excretion, UNOxV; *p<0.05 vs. respective SED. $^{+}$p<0.05 vs. respective Young.

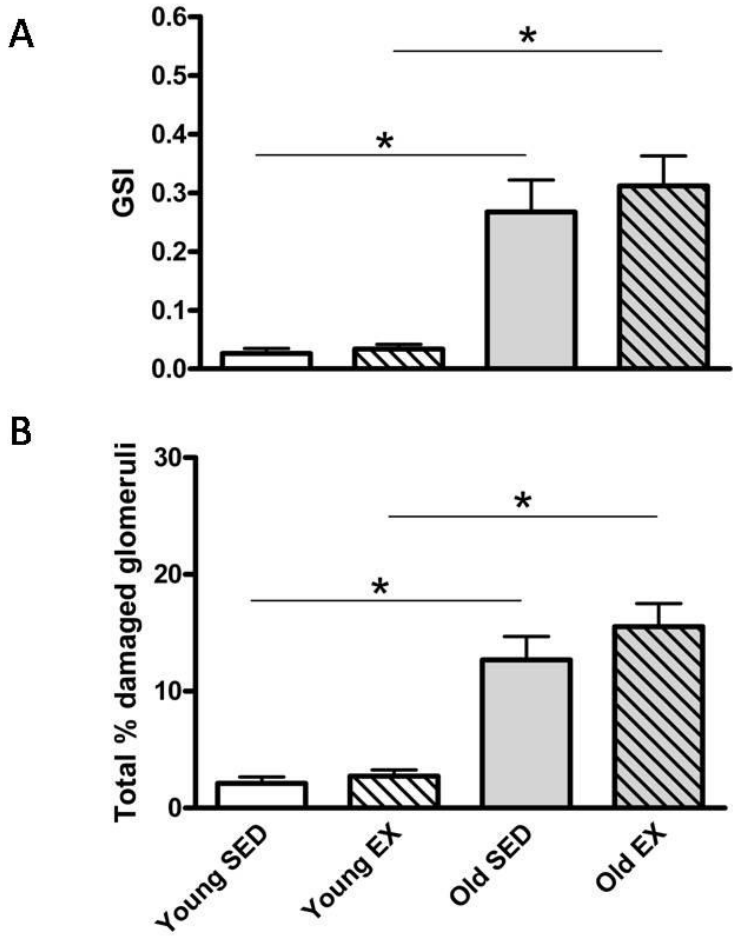

Figure 4-1. Glomerular structural changes in young and old, sedentary (SED) and exercise (EX) trained rats. Whole kidney sections were stained with periodic acid schiff, followed by a hematoxylin counterstain prior to blind histological grading. (A) The gomerular sclerosis index (GSI) significantly increased with age and was unaffected by 12 weeks of treadmill exercise (5 days/week, 60 min/day at 15 m/min, 15 degree incline). (B) The total percentage of damaged glomeruli also rose with age and exercise was without effect. *Denotes a statistical significance of $p<0.05$ between the two groups.

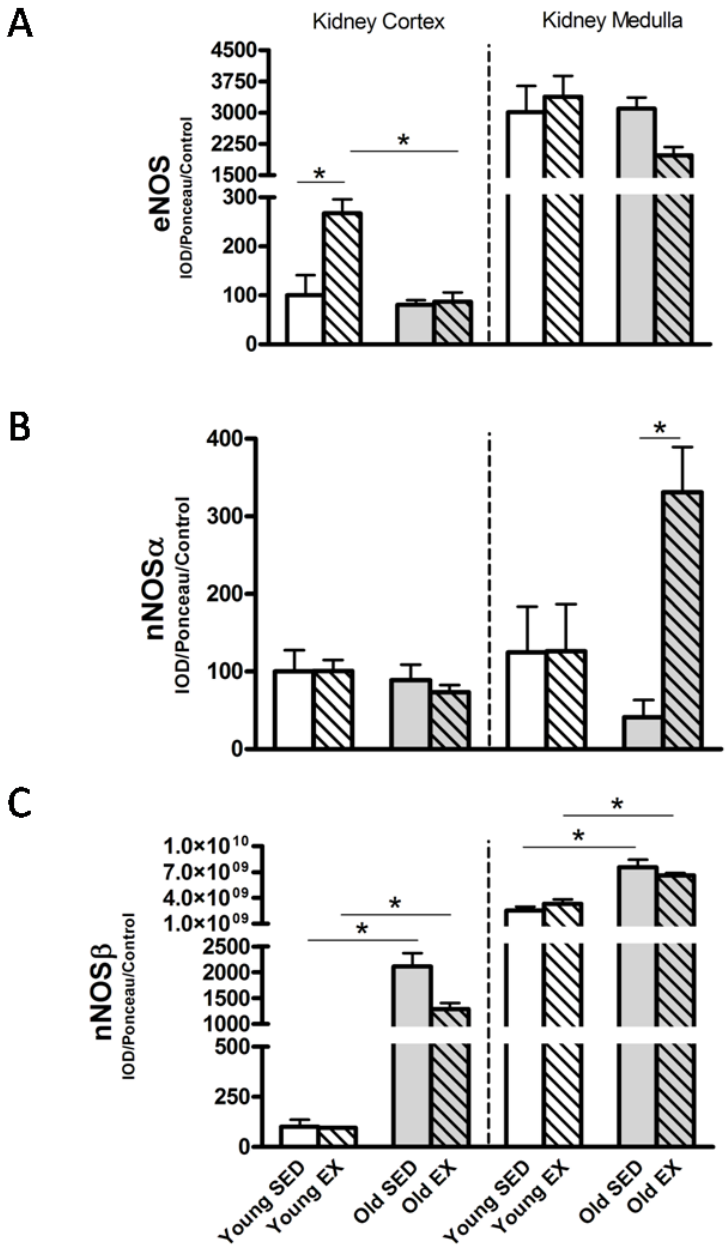

Figure 4-2. Protein levels of the nitric oxide synthase (NOS) enzymes in the kidney cortex and kidney medulla. Total homogenates of kidney cortex and kidney medulla from exercise (EX) trained and sedentary (SED) rats were run using Western blot and probed for (A) endothelial nitric oxide synthase (eNOS), (B) neuronal NOS isoform α (nNOSα), and (C) nNOS isoform β (nNOS β). Relative density units were expressed as a % from Young SED controls of the kidney cortex. *Denotes a statistical significance of $p<0.05$ between the two groups.

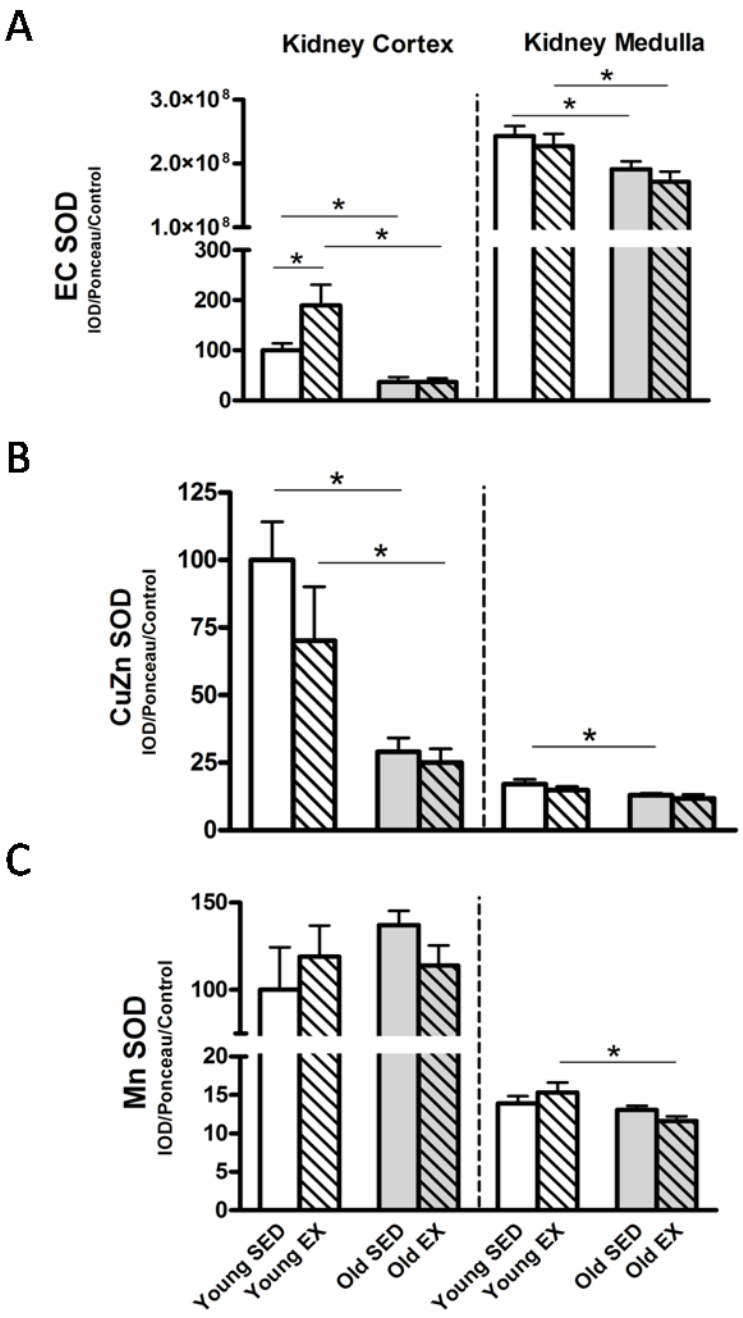

Figure 4-3. Protein levels of the superoxide dismutase (SOD) enzymes in the kidney cortex and kidney medulla. Total homogenates of kidney cortex and kidney medulla from exercise (EX) trained and sedentary (SED) rats were run using Western blot and probed for (A) extracellular superoxide dismutase (EC SOD), (B) cytosolic-localized copper/zinc SOD (CuZn SOD), and (C) mitochondrial-localized manganese SOD (Mn SOD). Relative density units were expressed as a % from Young SED controls of the kidney cortex. *Denotes a statistical significance of $p<0.05$ between the two groups.

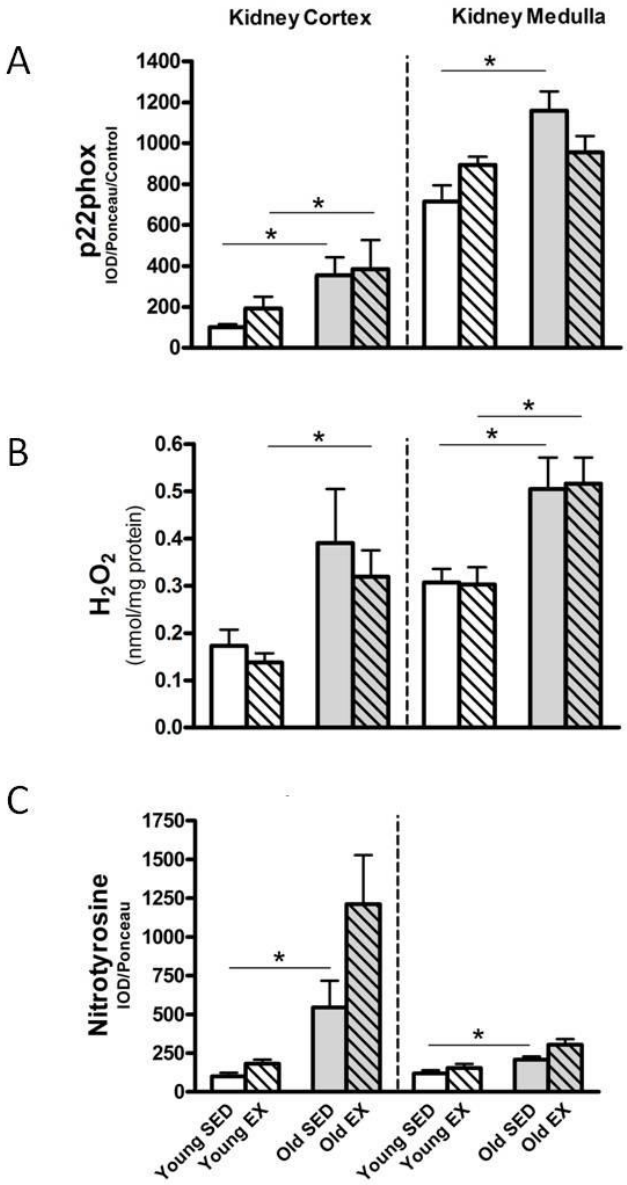

Figure 4-4. Oxidative stress markers: p22phox protein, H_2O_2 levels, and nitrotyrosine protein in kidney cortex and kidney medulla. Total homogenates of kidney cortex and kidney medulla from exercise (EX) trained and sedentary (SED) rats were prepared for (A) p22phox Western blot detection, (B) H_2O_2 concentration levels, and (C) nitrotyrosine Western blot detection. All values are expressed as a % from Young SED controls of the kidney cortex. *Denotes a statistical significance of $p<0.05$ between the two groups.

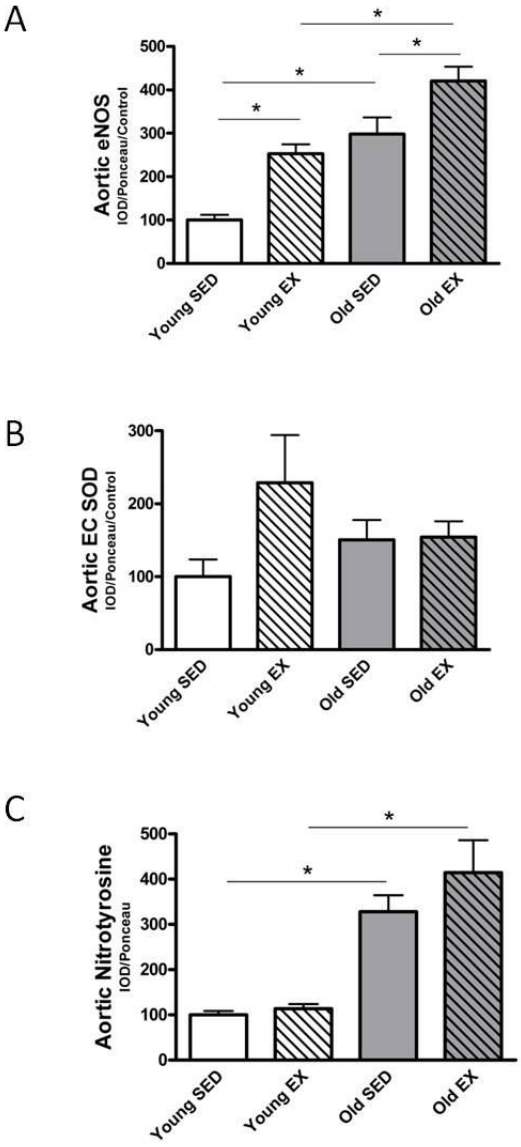

Figure 4-5. Aortic protein levels of eNOS (endothelial nitric oxide synthase), EC SOD (extracellular superoxide dismutase), and nitrotyrosine. Total homogenates of aorta from exercise (EX) trained and sedentary (SED) rats were run using Western blot and probed for (A) endothelial nitric oxide synthase (eNOS), (B) extracellular superoxide dismutase (EC SOD), and (C) nitrotyrosine . Relative density units were expressed as a % from Young SED controls of the kidney cortex. *Denotes a statistical significance of $p<0.05$ between the two groups.

CHAPTER 5
PROTECTION AGAINST AGE-DEPENDENT RENAL INJURY IN THE F344XBROWN NORWAY MALE RAT IS ASSOCIATED WITH MAINTAINED NITRIC OXIDE SYNTHASE

Background

In man, the kidney develops structural damage with age that is associated with thickening of the glomerular basement membrane, expansion of glomerular mesangium, increases in extracellular matrix proteins and appearance of tubulointerstitial injury (Kasiske, 1987; McLachlan *et al.* 1977). In addition, glomerular filtration rate (GFR) falls secondary to both glomerular injury and to falls in renal plasma flow because of renal vasoconstriction (McLachlan *et al.* 1977). Even in the absence of primary kidney disease, a decline in renal function is expected although not inevitable, as demonstrated by the Baltimore Longitudinal Study on Aging (Lindeman *et al.* 1985). Age-dependent kidney damage and dysfunction are also seen in the aging rat, with some strains showing rapidly developing, age-dependent chronic kidney disease (CKD), while others maintain excellent renal function and structure even when very old (Baylis & Corman, 1998).

All forms of CKD are associated with nitric oxide (NO) deficiency, which is both a result of CKD and a contributing factor to progression (Baylis, 2008; Baylis, 2009). In the Sprague-Dawley (SD) rat where renal disease progresses rapidly, age-dependent kidney damage is related to decreased abundance and activity of the NO synthesizing enzyme, NO synthase (NOS) in the kidney cortex (Erdely *et al.* 2003). Plasma levels of the endogenous inhibitor of NOS, asymmetric dimethylarginine (ADMA) are also elevated in elderly humans and rats (Boger *et al*. 2000; Kielstein *et al*. 2003; Xiong *et al.* 2001), providing an additional mechanism of NO deficiency in aging.

It is evident that genetic background plays a critical role in how organ function changes with age. In fact, age-dependent changes in humans can be attributed to more than 600 genes, about 100 of which contain expression-associated single nucleotide polymorphisms (Wheeler *et al.* 2009). In contrast to the injury prone Sprague-Dawley, age dependent CKD develops slowly in the Munich-Wistar rat and is minimal in WAG/Rij (Baylis & Corman, 1998) and Fisher 344xBrown Norway (F344xBN) strains (Lipman *et al.* 1996). With a life span of ~36 months and relatively preserved renal function, the F344xBN is considered a model of "healthy aging" (Diz, 2008). When compared to another commonly utilized aging model, the F344, which interestingly has increased insulin resistance and glomerular nephropathy but no systemic hypertension with age, the F344xBN has fewer glomerular lesions and a greater mean age at which 50% mortality occurs (F344: 103 wks. vs. F344xBN: 145 wks; Lipman *et al.* 1996). It is clear that genetic differences dictate outcome of age-related declines so further characterization of the various aging models is critical for aging investigations.

In the present study, we investigated the impact of aging on various determinants of NO production in the F344xBN rat. Determinants included 1) abundance of the NO synthesizing protein, NOS; 2) NOS inhibitor levels; 3) abundance of enzymes that regulate NOS inhibitors; 4) abundance of anti-oxidant and oxidative markers. The primary goal was to test the hypothesis that in the absence of significant age-dependent damage, renal NOS abundance is maintained. We also investigated whether there would be any beneficial effect of chronic renin-angiotensin system (RAS) blockade in these "protected" rats.

Methods

Animal Procedures

All animal procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (*Principles of Laboratory Animal Care*; NIH publication No. 86-23, revised 1985) and approved and monitored by the University of Florida Institutional Animal Care and Use Committee. Young (3 months; n=8) and old (24 months; n=24) male F344xBN rats were purchased from the National Institute of Aging colony (Harlan Sprague Dawley, Indianapolis, IN) and singly housed in a temperature/light-controlled environment and given access to standard rat chow/water *ad libitum*. Old rats were divided into three groups (n=8/group) and used to compare 6 months of placebo (normal aging) with RAS blockade with either an angiotensin converting enzyme inhibitor (ACEI; enalapril; 40 mg/kg body weight) or angiotensin II receptor blocker (ARB; candesartan; 10 mg/kg body weight). Bacon flavored tablets (BioServ #F05072) were given with or without the drug compounded into them, and old rats were sacrificed for study at 30 months of age by rapid decapitation. Young rats (n=8) were maintained on the same *ad lib* diet and daily bacon flavored tablets without drug for two weeks and then sacrificed at ~3 months of age under isoflurane anesthesia. Blood was taken either by aortic puncture or from the trunk in young and old, respectively, and then spun for collection of plasma. The kidneys were removed and while one kidney was prepared for histological analyses (see below), the other was separated into cortical and medullary sections, and then flash-frozen in liquid nitrogen. However, all analyses were conducted in cortical tissue only. All samples were stored at -80°C for further analyses. Plasma creatinine levels were measured by HPLC as previously described (Sasser *et al.* 2009).

Renal Pathology

One kidney was cut along the transverse axis and fixed in 10% buffered formalin for 48 hours at 4°C, paraffin wax embedded, cut into 5-micron thick sections and stained with periodic acid schiff, followed by a hematoxylin counterstain (Sigma). Sections were then examined, blind, for the level of glomerular sclerosis, glomerular ischemia/atrophy, tubular atrophy, and interstitial fibrosis. Each category was scored (0=none, 1=10%, 2=10–25%, 3=25–50%, 4=50–75%, 5=75–100%) based on the percentage of structures that displayed the described injury.

Western Blot

Relative protein abundances of endothelial nitric oxide synthase (eNOS; BD Transduction; 1:250), neuronal NOSα (nNOSα; Santa Cruz; 1:50), nNOSβ (ABR; 1:500), dimethyldiaminohydrolase (DDAH) isoforms (Santa Cruz; DDAH1 1:250 and DDAH2 1:100), protein methyltransferase (PRMT1; Millipore; 1:2000), superoxide dismutase (SOD) isoforms (Stressgen Reagents; EC SOD 1:250, CuZn SOD 1:2000, and Mn SOD 1:2000), and p22phox (Santa Cruz; 1:50) were measured by Western Blot. Homogenized samples of kidney cortex standardized by protein concentrations (50-200 ug) were separated by electrophoresis (7.5% or 12% acrylamide gel, 200 V, 65 min) and transferred onto nitrocellulose membranes (GE Healthcare) for 60 min at 0.18 A as previously described (Sasser *et al.* 2009). Membranes were stained with Ponceau Red (Sigma) to check for transfer efficiency/uniformity and equal loading, incubated in blocking solution for 60 min, and then washed in TBS + 0.05 % Tween before overnight primary antibody incubation at 4°C. Membranes were then incubated with the appropriate secondary antibody for one hour at room temperature, with a series of washes before and after, and developed with enhanced chemiluminescent reagents

(Thermo Scientific). Bands were quantified by densitometry using the VersaDoc™ Imaging System and One Analysis Software (BioRad). Protein abundance was calculated as integrated optical density (IOD) of the protein of interest (after subtraction of background), factored for Ponceau Red stain (total protein loaded), and normalized with an internal positive control value. This allowed for quantitative comparisons between different membranes. The specific protein abundance is represented as IOD/Red Ponceau/Control relative to the appropriate control group.

Analytical Methods

ADMA concentrations in plasma and renal cortical tissue homogenates were measured by reverse-phase HPLC using the Waters AccQ-Fluor fluorescence method as previously described (Sasser *et al.* 2009). Renal cortical tissue ADMA concentrations (μM) were normalized to total protein (mg/mL) and therefore expressed as μmol/mg. Renal cortical concentration of hydrogen peroxide was measured using the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes) according to the manufacturer's instructions with the following modifications: Renal cortical tissue was homogenized with 1x phosphate-buffered saline (PBS; MediaTech, Inc.; 0.1 g tissue: 400 μL PBS), diluted 1:3, incubated at room temperature with Amplex® Red reagent and HRP for 45 minutes, and then read at a wavelength absorbance of 560 nm. Assay specificity was confirmed using 2000 units of catalase (Sigma). Renal cortical H_2O_2 concentrations (μM) were normalized to total protein (mg/mL) and therefore expressed as nmol/mg.

Statistical Analyses

Data are presented as mean ± SEM and analyzed by either one-way ANOVA followed by post-hoc analysis, or the non-parametric Kruskal-Wallis test (for histology

only) using GraphPad Prism 4 software (San Diego, CA). Significance was defined as $p<0.05$.

Results

Body weight (BW) increased with age (young: 337±8 vs. old: 560±24 g) and plasma creatinine fell (young: 0.11±0.01 vs. old: 0.06±0.00 mg/dl). Histological analyses revealed no difference in the percentage of glomeruli with sclerosis in old vs. young rats although there were increases in glomerular ischemia/atrophy, tubular atrophy, and interstitial fibrosis with age (Fig. 5-1). RAS blockade had no impact on the age-dependent rise in BW (old: 560±24 vs. old-ACEI: 542±17 vs. old-ARB: 546±18 g) but plasma creatinine significantly rose with both ACEI and ARB treatment (old-ACEI: 0.09±0.01 vs. old-ARB: 0.09 vs. 0.01 mg/dl) in comparison to untreated old rats. The only impact of RAS inhibition on histology was that ARB treatment significantly reduced both tubular atrophy and interstitial fibrosis compared to untreated old rats and those given ACEI (Fig. 5-1).

NOx (stable metabolites of NO=NO3 + NO2) levels in the plasma were unchanged with age (young: 12±1 vs. old: 12±4 μM) and ARB treatment (9±2 μM), although ACEI increased plasma NOx (30±1 μM). Both eNOS and nNOSα were present in increased abundance in the kidney cortex of old rats (Figs. 5-2*A* and *B*), whereas nNOSβ abundance (Fig. 5-2*C*) remained unchanged. Neither ACEI nor ARB treatment in old rats affected kidney cortex eNOS or nNOSβ abundance (Figs. 5-2*A* and *C*); however, ARB did increase nNOSβ abundance compared to old untreated rats and marked increases in nNOSα were detected with both ACEI and ARB treatment (Fig. 5-2*B*).

Plasma levels of ADMA, L-Arginine, and SDMA were unchanged with aging although the plasma L-Arginine:ADMA ratio increased, favoring increased NO production (Table 5-1). Similarly, in the kidney cortex there were also no differences in ADMA and L-Arginine levels in young vs. old, although the L-Arginine:ADMA ratio increased (Table 5-2). There was no effect of RAS blockade on plasma levels of ADMA, L-Arginine, L-Arginine:ADMA ratio, or SDMA compared to old untreated rats (Table 5-1). Kidney cortex PRMT-1, the enzyme responsible for ADMA production was increased with age (Fig. 5-3*A*) whereas the ADMA-degrading enzyme, DDAH1 tended to increase in abundance (Fig. 5-3*B*) and there was no change in DDAH2 abundance in the kidney cortex (Fig. 5-3*C*). ACEI and ARB treatment reduced kidney cortex PRMT-1 abundance (Fig. 5-3*A*) but had no effect on abundance of either DDAH isoform compared to kidneys from old untreated rats (Figs. 5-3B and *C*).

We also assessed indices of oxidative stress in the F344xBN rodent model of aging and found significant increases in p22phox abundance in the kidney cortex of old vs. young rats (Fig. 5-4*A*) but no change in the H_2O_2 conμμtent of the kidney cortex (Fig. 5-4*B*). Old rats showed marked increases in the kidney cortex abundance of EC SOD and Mn SOD with no change in CuZn SOD abundance (Fig. 5-5). RAS blockade had no impact on kidney cortex p22phox abundance or H_2O_2 content compared to old untreated rats (Figs. 5-4*A* and *B*), although ACEI reduced kidney cortex EC SOD abundance to levels that fell further with ARB (Fig. 5-5*A*). In contrast, ACEI increased CuZn SOD abundance whereas ARB decreased it (Fig. 5-5*B*). No statistically significant changes were detected for the Mn SOD abundance among any of the old groups (Fig. 5-5*C*).

Discussion

The main finding of this study is that the protection against age-dependent glomerular sclerosis seen in the F344xBN rat is associated with preserved eNOS and nNOSα protein abundance in kidney cortex and with no age-dependent increase in plasma ADMA. Also, the antioxidant SOD's in the kidney increased with age and apparently balanced the increased oxidant-generating NADPH oxidase (indicated by increased p22phox). Although this rat strain does not develop glomerular sclerosis with age, tubulointerstitial injury increased, which was prevented by RAS inhibition with the ARB, whereas ACEI had no protective effect.

It is always difficult to determine what changes occur due to normal aging and what is due to increased susceptibility to acquired disease. The renal response to aging is extremely variable in humans and in rats (Baylis & Corman, 1998; Lindeman *et al.* 1985), and of note, the female of many strains, including the SD, show marked protection compared to the male (Baylis & Corman, 1998; Erdely *et al.* 2003). In the aged male SD rat where glomerular sclerosis is severe (>60% of glomeruli damaged at 24 months), renal eNOS and nNOS protein abundance falls with age (Erdely *et al.* 2003). In contrast, in the present study we report that in the aged male F344xBN, where only ~ 2.5% of glomeruli show sclerotic damage by 30 months of age, the renal eNOS and nNOS isoforms are preserved. We have suggested that falls in renal NOS protein abundance are both a consequence and a cause of progression of several forms of CKD (Baylis, 2009). The present study expands this by showing that normal aging is not inevitably accompanied by loss of renal NOS, reinforcing our hypothesis that there is a causal association between kidney injury and loss of renal NOS protein (Baylis, 2009).

In addition to NOS protein abundance, NO production is controlled by the local and circulating concentrations of the endogenous NOS inhibitor, ADMA. Several studies report that plasma ADMA increases in normal aging humans and that an increase, although delayed, also occurs in aging women (Kielstein *et al.* 2003; Schulze *et al*. 2005). This may be associated with the age-dependent development of endothelial dysfunction (Bode-Boger *et al.* 2003). Increased plasma ADMA has also been reported in the aging male SD rat (Xiong *et al.* 2001). Control of plasma ADMA level is mainly by degradation by the DDAH1 enzyme, which is abundant in liver and kidney (Palm *et al.* 2007; Sasser *et al.* 2009). ADMA is made by PRMT1, a class of enzymes that methylate amino acids (including arginine) while they are incorporated into intact proteins (Nicholson *et al.* 2009). The free (active) ADMA is released by proteolysis. In this study, we found that in the 30-month old male F344xBN, neither circulating nor renal cortical ADMA levels were changed with age. The renal abundance of the enzyme responsible for ADMA production, PRMT-1, was unchanged with age and the ADMA-degrading enzyme, DDAH1 (main renal enzyme) tended to increase and DDAH2 was unchanged. Thus, an increase in plasma ADMA with age is not inevitable and in fact we observed an *increase* in the ratio between L-Arginine/ADMA, an effect that favors NO production and enhanced endothelial function. This was due to a non-significant tendency for increased plasma L-Arginine with age, also reported by us previously in the aging male SD (Mistry *et al.* 2002).

Furthermore, in spite of a lifetime (30 months) of oxidative metabolism, the F344xBN shows no obvious signs of increased oxidative stress. DDAH activity and abundance is reported to be redox sensitive and it is widely believed that exposure to

oxidative stress will inhibit DDAH and increase ADMA (Palm *et al.* 2007). Activity of PRMT1 is also increased by oxidative stress; however, as noted above, the abundance of these enzymes was not affected by aging in the F344xBN. Ang-II induces oxidative stress in the kidney (Gill & Wilcox, 2006; Modlinger *et al.* 2006) and when kidney injury develops with age, there is also activation of the intrarenal angiotensin II (Ang II) system; however in the F344/BN rat where no glomerular damage develops, there is no increase in intrarenal ANGII (Kasper, 2008). We did observe an increased abundance of the renal NADPH oxidase subunit, p22phox, with age, which presumably implies increased superoxide production. However, we also found increases in EC SOD and Mn SOD, a likely compensatory response to scavenge the increased superoxide production. Since renal cortical H_2O_2 is also unchanged with age there is presumably also increased renal catalase activity.

Chronic RAS blockade is used for treatment of CKD and/or hypertension. Chronic ACEI protects against glomerular sclerosis, reduces proteinuria, and lowers blood pressure in aging male Munich-Wistar rats, treated from 3 to 30 months of age (Anderson *et al.* 1994). Lifetime treatment with ACEI in the male WAG/Rij rat also reduces blood pressure, urinary protein excretion, and expansion of the mesangial matrix (Heudes *et al.* 1994), although WAG/Rij rats show minimal age-dependent injury (Baylis & Corman, 1998). In the present study, male F344xBN rats show little evidence of glomerular sclerosis and yet some beneficial effects of RAS blockade were observed. It is interesting that even in the absence of glomerular sclerosis, significant tubulointerstitial injury develops in these rats by 30 months of age, and 6 months of treatment with the ARB protected against tubulointerstitial injury. Tubulointerstitial injury

develops as peritubular capillaries are lost in various forms of CKD and is particularly prominent in slowly evolving age-dependent damage (Lombardi *et al.* 1999).

Although not reported in the present study, there is no age-dependent proteinuria in the F344xBN rat, and long term ACE inhibition with enalapril had no effect on urinary protein excretion (Kasper, 2008). Thus, tubulointerstitial injury, in the absence of glomerular injury, does not cause proteinuria in this model. In the aging Wistar rat, where substantial glomerular damage occurs, endothelin receptor blockade reversed proteinuria and prevented glomeruloslcerosis, while tubulointerstitial injury remained (Ortmann *et al.* 2004). Thus, it is likely that the development of proteinuria in response to kidney damage is primarily glomerular, rather than tubular in origin.

Tubulointerstitial injury has also been suggested to be an NO deficiency-mediated event in the aging kidney (Lombardi *et al.* 1999). Although there is no evidence of renal NO deficiency in the 30-month old F344xBN male, both ARB and ACEI lead to marked increases in renal cortical nNOSα in the present study. However, in contrast to the beneficial effect of ARB, 6 months of ACEI treatment gave no reduction in tubulointerstitial injury, suggesting that the protection against tubulointerstitial injury produced by the ARB is not due to nNOSα stimulation. It is interesting that in this setting the ARB is superior to ACEI, whereas ACEI outperforms ARB in heart failure (Berry *et al.* 2001). ARB and ACEI are also thought to have equivalent efficacy in treating patients with a wide range of cardiovascular risk (Baumhakel & Bohm, 2009; Schindler, 2008).

The fall in plasma creatinine in the old untreated rat most likely relates to the well-known loss of lean body mass that occurs with age (Griffiths, 1996). It is interesting that

both methods for RAS blockade raised plasma creatinine to the young (still very low) value. This may reflect RAS blockade-induced changes in body composition and/or activity level. It is unlikely to reflect loss of renal function since chronic intrarenal RAS blockade is associated with beneficial effects. Further, the plasma SDMA is unaffected by aging or RAS blockade and SDMA normally increases as GFR falls (Marcovecchio *et al.* 2009).

In summary, we conclude that in the aged male F344xBN rat, in contrast to our previous findings in the aged male SD rat, renal NOS is preserved and there is minimal age-dependent glomerular sclerosis (Erdely et al. 2003). Both anti-oxidant and oxidant systems in the kidney are activated with age and there is no net effect on circulating or renal cortical ADMA concentrations. The tubulointerstitial injury seen with aging is reversed with 6 months of ARB but not ACEI, and is not associated with renal NOS. Our data highlight the complexity of the aging process in that factors such as genetic background can dictate histological outcomes that are associated with the renal NO system.

Table 5-1. The effect of aging and chronic RAS blockade on plasma levels of ADMA, L-Arginine, L-Arginine:ADMA, and SDMA in young (~3 month), old (30 month), old-ACEI, and old-ARB male F344xBN rats.

Group	ADMA (μM)	L-Arginine (μM)	L-Arginine:ADMA	SDMA (μM)
Plasma -Young	0.26±0.02	89±7	346±14	0.18±0.02
Plasma - Old	0.26±0.04	102±9.0	430±34*	0.20±0.02
Plasma – Old ACEI	0.30±0.13	106±30	442±30	0.22±0.07
Plasma – Old ARB	0.21±0.03	94±26	374±33	0.19±0.04

RAS, renin angiotensin system; ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker. ACEI and ARB treatment lasted for 6 months in old only. *p<0.05 vs. young.

Table 5-2. The effect of aging on kidney cortex levels of ADMA, L-Arginine, L-Arginine:ADMA, and SDMA in young (~3 month) and old (30 month) male F344xBN rats.

Group	ADMA (nmol/g)	L-Arginine (nmol/mg)	L-Arginine:ADMA	SDMA (nmol/g)
Kidney Cortex -Young	9±1	2.00±0.32	216±25	28±2
Kidney Cortex - Old	8±1	2.00±0.18	298±24*	22±2*

RAS, renin angiotensin system; ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker. ACEI and ARB treatment lasted for 6 months in old only. *p<0.05 vs. young.

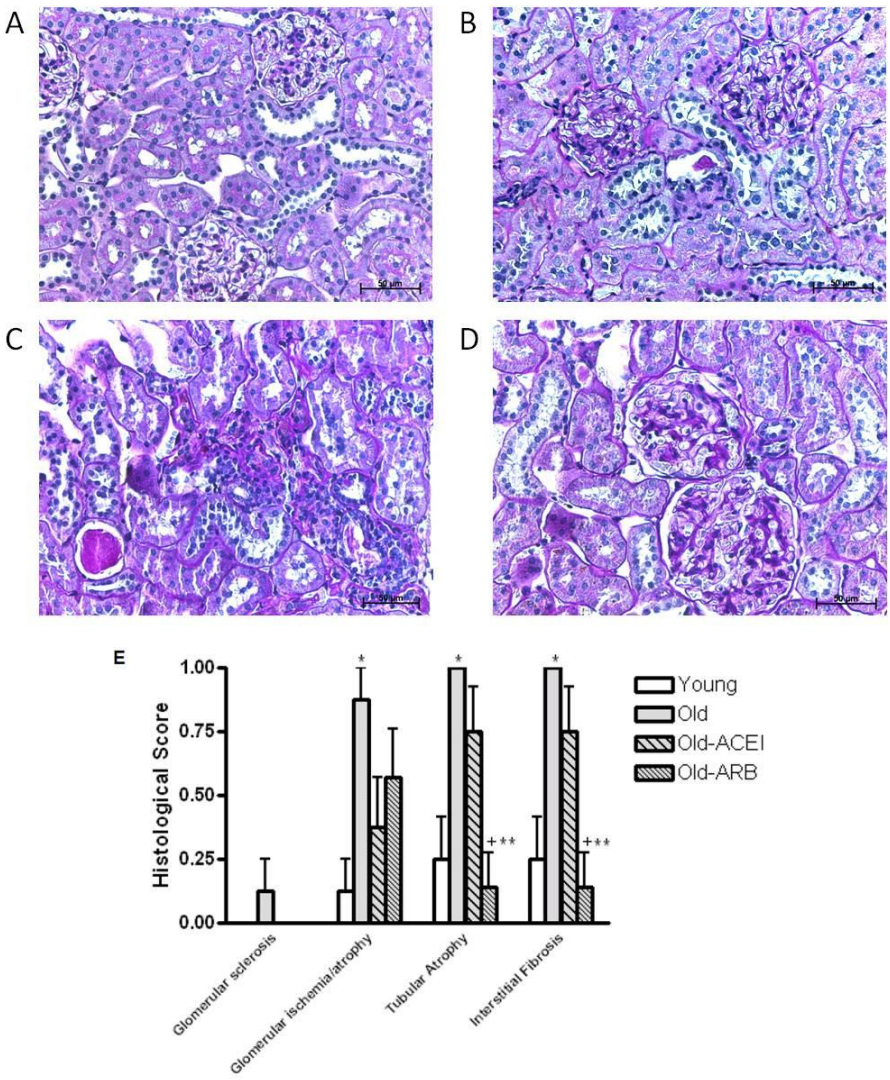

Figure 5-1. Representative images of periodic acid Schiff stained sections that were examined, blind, for the level of glomerular sclerosis, glomerular ischemia/atrophy, tubular atrophy, and interstitial fibrosis. (A) A healthy, normal cortex in the young rat (~3 months). (B) A 30-month old, untreated rat, with mild glomerular sclerosis (left glomerulus) and tubular atrophy. (C) A 30-month old rat given 6 months of angiotensin converting enzyme inhibition (old-ACEI) with focal interstitial fibrosis (center of image). (D) A 30-month old rat given 6 months of angiotensin receptor blockade (old-ARB) showing some mesangial matrix expansion but otherwise with preserved glomerular and tubular architecture. (E) Quantification of the histological data. Each category was scored (0=none, 1=10%, 2=10–25%, 3=25–50%, 4=50–75%, 5=75–100%) based on the percentage of structures that displayed the described injury. *p<0.05 vs. Young; +p<0.05 vs. Old; **p<0.05 vs. Old-ACEI; n=8/group for all.

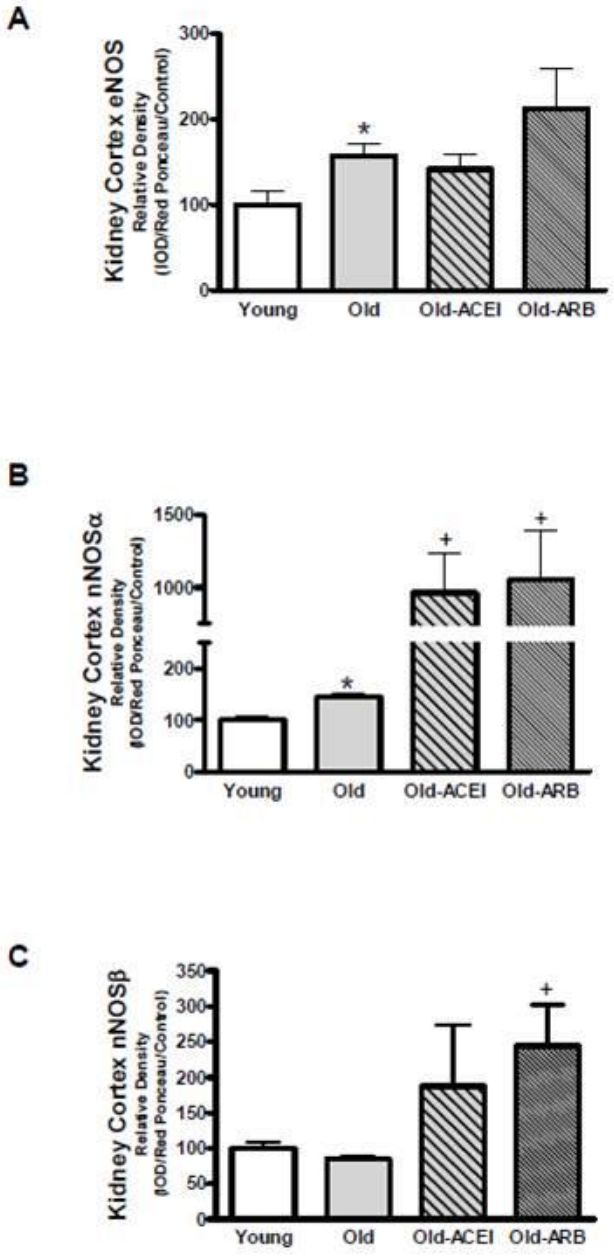

Figure 5-2. We assessed the kidney cortex protein abundance of the nitric oxide synthase (NOS) enzymes in young (~3 month), old (30 month), old-ARB, and old-ACEI male F344xBN rats: (A) endothelial (e)NOS (A), (B) neuronal (n) NOS isoform α (nNOSα), and (C) nNOS isoform β (nNOS β). *p<0.05 vs. young; +p<0.05 vs. old; n=8/group for all. Relative densities in protein abundance were determined by Western blot and normalized to Red Ponceau staining and an internal positive control.

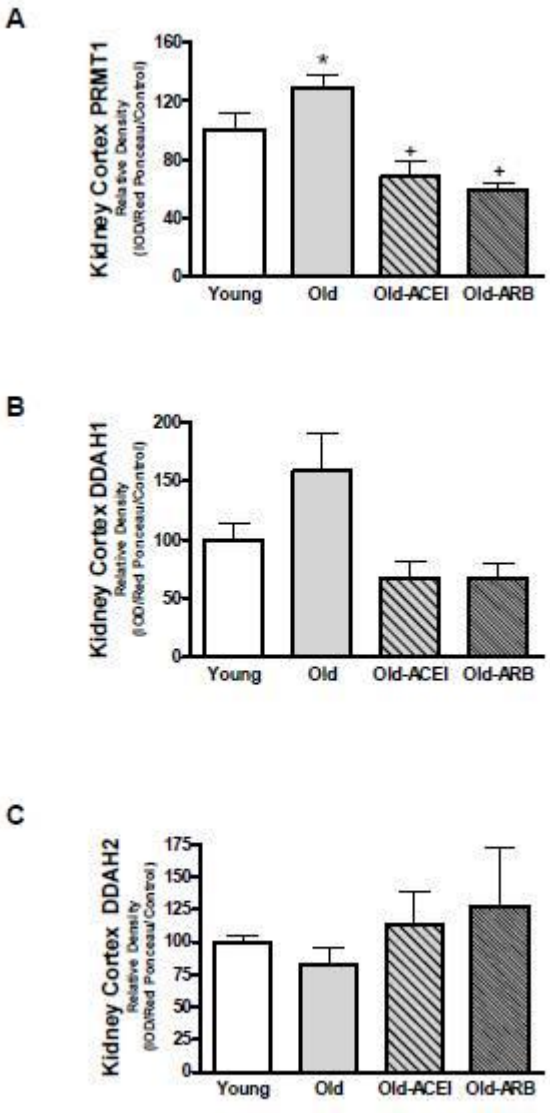

Figure 5-3. We assessed the kidney cortex protein abundance of the enzymes that regulate asymmetric dimethylarginine (ADMA) synthesis and catabolism in young (~3 month), old (30 month), old-ARB, and old-ACEI male F344xBN rats: (A) Protein methyltransferase-1 (PRMT-1), (B) dimethyldiaminohydrolase1 (DDAH1), and (C) DDAH2. *p<0.05 vs. young; +p<0.05 vs. old; n=8/group for all. Relative densities in protein abundance were determined by Western blot and normalized to Red Ponceau staining and an internal positive control.

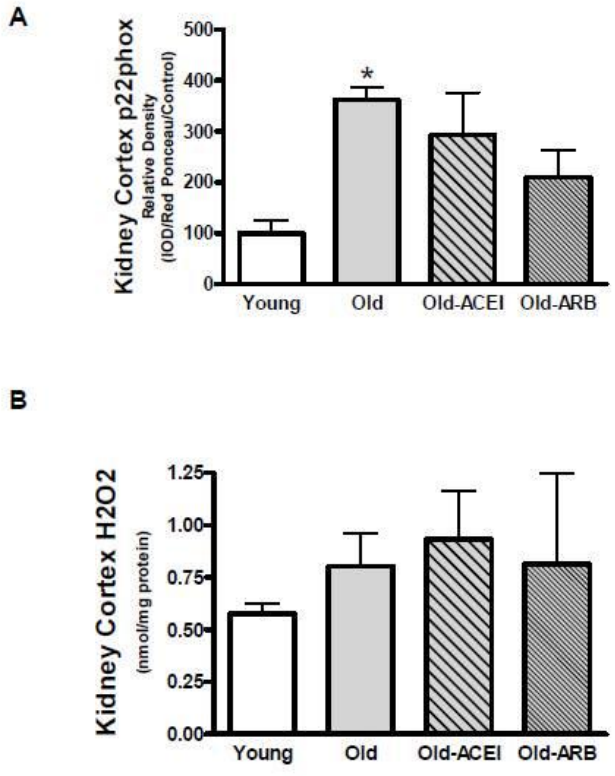

Figure 5-4. Oxidative stress in the kidney cortex as assessed by determining the protein abundance of p22phox (A) and content H_2O_2 (B) in young (~3 month), old (30 month), old-ARB and old-ACEI male F344xBN rats. *p<0.05 vs. Young; n=8/group for all. Relative densities in protein abundance were determined by Western blot and normalized to Red Ponceau staining and an internal positive control. Kidney cortex H_2O_2 content was confirmed with catalase and normalized to mg of protein.

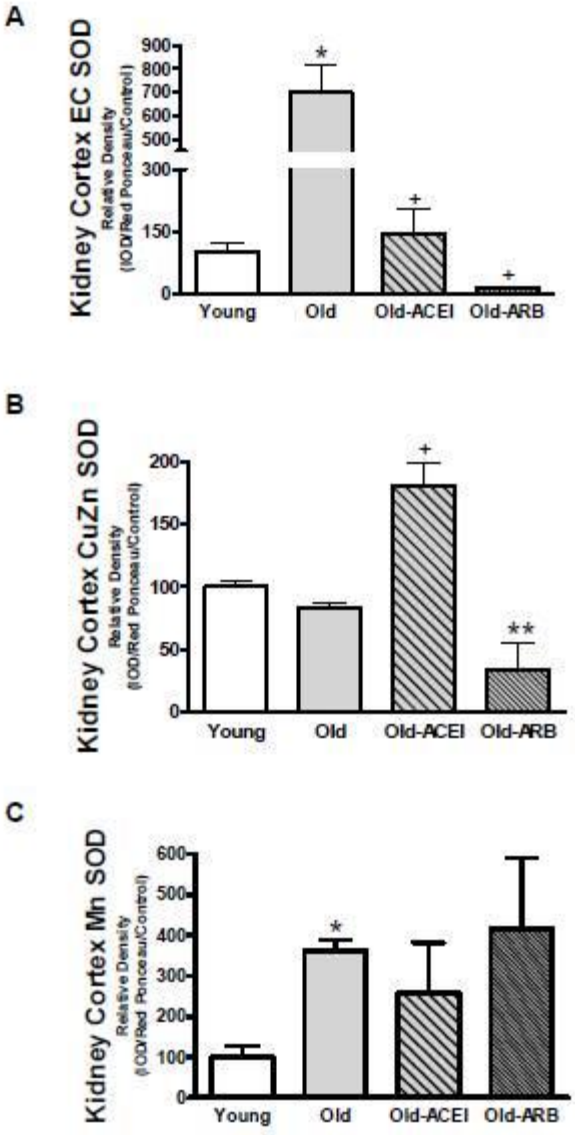

Figure 5-5. We assessed the kidney cortex protein abundance of the antioxidant enzymes in young (~3 month), old (30 month), old-ARB, and old-ACEI male F344xBN rats: (A) Extracellular superoxide dismutase (EC SOD), (B) cytosolic-located CuZn SOD, and (C) mitochondrial-located Mn SOD (Mn SOD). *p<0.05 vs. Young; +p<0.05 vs. Old; **p<0.05 vs. Old-ACEI; n=8/group for all. Relative densities in protein abundance were determined by Western blot and normalized to Red Ponceau staining and an internal positive control.

CHAPTER 6
CONCLUSIONS

This primary purpose of this body of work is to characterize the impact of exercise on the renal NO and antioxidant systems in states of health and injury. The overall hypothesis is that the reduction in blood flow to the kidney during exercise decreases the shear stress-dependent enzymes eNOS and EC SOD which are required for optimal vascular health. This may put the kidney at risk for falls in NO and antioxidant bioavailability. Indeed, it has been shown that exercise reduces renal eNOS and/or NOS activity (Miyauchi *et al.* 2003; Lin *et al.* 2010). The studies previously described in this body of work demonstrate that the renal response to exercise and age are influenced by genetic background. Further, exercise also influences the status of pre-existing renal endothelial health which determines severity of IR-induced AKI. Consideration of these factors is required for optimal exercise benefit for patients with endothelial dysfunctional including but not limited to renal disease. In this section, a summary of findings directly taken from each study will be presented followed by a general discussion.

Summary of Findings

Genetic Background Determines Renal Response to VWR EX

The protein abundance of eNOS and EC SOD in response to 12 weeks of VWR EX is variable and influenced by genetic background. Despite comparable running profiles and equivalent renal functional responses to 6 and 12 weeks of VWR, we detected profound differences in the kidney cortex's response to exercise between young adult male SD and F344 rats. In the SD rat, VWR significantly decreased kidney cortex eNOS, EC SOD and p22phox whereas in the F344, VWR increased these

variables. Immunohistochemical studies confirmed that the strain dependent changes in eNOS occur exclusively in the vascular endothelium. These directionally opposite changes in eNOS and EC SOD abundance between the two rat strains suggest that while chronic mild exercise may have beneficial renal vascular effects in the F344, it could be damaging to the SD.

Genetic Background Determines Susceptibility to IR-induced AKI

Despite differences in renal eNOS and EC SOD responses to VWR EX between the SD and F344 rat, lung eNOS and EC SOD for either strain both increased with VWR EX and IR injury. We also found that 12 weeks of VWR EX exacerbated susceptibility to IR-induced AKI in the SD but not the F344 rat. In both strains, the reduction in RBF with acute exercise in the untrained SED rat was lost in the trained rat (10-12 weeks of TM EX, 15 m/min). Resting RBF values fell similarly in the trained rats of each strain. Our data indicate that vulnerability to an oxidative-stress mediated renal insult such as IR-induced AKI is determined by the state of endothelial health which is influenced by genetic background. These strain differences are not attributable to renal hemodynamic responses to TM EX.

Chronic TM EX Does Not Alter Age-Related Renal Injury

10-12 weeks of TM EX increased both eNOS and EC SOD abundance in the kidney cortex of the young rat, despite an expected exercise-induced fall in RBF. There was no change in renal eNOS and EC SOD with exercise. Exercise did not reverse age-dependent renal damage. Whereas nNOSβ markedly increased, there was no effect on either eNOS or nNOS∝ in the kidney cortex with age. TM EX did not influence an age-dependent loss of the kidney cortex antioxidants EC SOD and CuZn SOD and age-dependent increase with p22phox. Aging F344 rats also developed significant

proteinuria with some loss of renal function and a tendency for total NO production to fall, and that exercise does not reverse these decremented changes. These data suggest that long-term TM EX does not alter age-associated renal injury in the male F344 rat.

Protection Against Age-Related Renal Damage Associates with Intact NO System

In the F344xBN rat, a lack of development of glomerular sclerosis with age associated with increased kidney cortex eNOS and nNOSα and no change in plasma ADMA. The antioxidant SOD's also increased in the kidney cortex with age despite an increase in the p22phox subunit of the superoxide generating NADPH oxidase. Although this rat strain did not develop glomerular sclerosis with age, tubulointerstitial injury increased, which was prevented by RAS inhibition with the ARB candesartan, whereas the ACEI enalapril had no protective effect. These data suggest that protection against age-related renal injury associates with an intact NO system.

General Discussion

Although kidney function is critical for body fluid, electrolyte, and acid-base homeostasis, it is an organ system that is much less investigated in the field of exercise physiology. Many of the studies outlining the major functional responses were reported in very early investigations. Therefore, it is a main purpose of this work to contribute new understanding on the physiological responses of the kidney to exercise in states of health and disease.

Vascular benefits of exercise are mainly due to shear stress-dependent mechanisms. Shear stress, or the frictional force exerted on a vessel wall, potently stimulates NO production by increasing eNOS mRNA stability, eNOS transcription and translation, and eNOS enzyme activity (Harrison *et al.* 2006). NO is a critical regulator

of vasomotor tone and contains several anti-atherosclerotic properties including inhibition of vascular smooth muscle proliferation and platelet aggregation. In, addition, shear stress can also up-regulate several antioxidants including EC SOD (Harrison et al. 2006). Mice deficient in c-Src, a tyrosine kinase critical for the shear stress response, failed to increase their aortic eNOS and EC SOD protein levels with exercise training (Davis *et al.* 2003), demonstrating that the importance of blood flow, and therefore shear stress, dictates the vascular benefits of exercise.

However, despite increasing evidence for the beneficial role of NO in exercise hyperemia, its role in tissues where blood flow reduces is less understood. Reductions in RBF are required to provide working muscles increased blood flow during exercise and are determined by intensity which is further controlled by several factors including the renal sympathetic nervous system and vasoconstricting hormones such as angiotensin II and endothelin-1 (McAllister, 1998). At high intensities, GFR is severely compromised due to marked falls in RBF. However, physical conditioning reduces the magnitude of reduction in RBF (Armstrong & Laughlin, 1984). Nonetheless, it has been shown that in SD rats, exhaustive exercise leads to development of renal structural injury and to falls in renal NOS activity (Lin *et al.* 2010). In Wistar rats, acute treadmill exercise also significantly reduced renal eNOS mRNA, protein, and NOS activity, opposite to the increased renal eNOS protein content found in the lung (Miyauchi *et al.* 2003).

Studies reported in Chapter 2 and 3 demonstrated that VWR EX reduced renal eNOS and EC SOD in the SD rat, whereas in the F344 rat, these enzymes significantly increased. The responses in the F344 were not unique to VWR EX since we validated

increased renal eNOS and EC SOD with 10-12 weeks of TM EX (Chapter 4). Interestingly, we also detected decreased renal p22phox abundance, a marker of oxidative stress, in the SD rat, which again contrasted findings in the F344 rat. These were unexpected since we predicted falls in NO and antioxidant capacity in the SD to increase oxidative stress, and vice versa for the F344 rat. Nonetheless, findings of Chapter 3 where we superimposed AKI, revealed the functional importance of these findings.

It is well known that NO is critical for normal renal function and its deficiency is a cause and consequence of CKD (Baylis, 2007). Insults that promote falls in NO bioavailability can therefore render the kidney susceptible to injury, as in the case of our VWR EX-trained SD rats. Indeed, there are reports of renal ischemia leading to exercise-induced AKI (Seedat *et al.* 1990; Yan *et al.* 2010; Bosch *et al.* 2009). In addition, when there is pre-existing oxidative stress, development of exercise-induced AKI is high, for example, in patients with renal hypouricemia (Yan *et al.* 2010; Saito *et al.* 2011; Ishikawa, 2002). As described in Chapter 3, we discovered that severity of IR-induced AKI was exacerbated in the VWR-EX trained SD rat compared to SED SD controls. Reductions in GFR and RPF due to IR injury were more severe with VWR EX in the SD rat. In contrast, VWR EX conferred protection against development of IR injury in the F344 rat. Even without the influence of exercise, we discovered a strain difference in susceptibility to IR in SED rats with the F344 more vulnerable. UNX-IR reduced GFR by 63% and 87% in the SD and F344 rat, respectively. Moreover, there were also greater reductions in RPF with UNX-IR in the F344 rat versus SD rat (65% vs. 78%, SD vs. F344, respectively). However, despite greater reductions in renal function

to UNX-IR, exercise afforded protection in the F344 rat. These findings underscore the influence of genetic background in determining the impact of exercise on the kidney.

There are several known differences between the SD and F344 rat. However, to our knowledge, we are the first group to directly compare their renal responses to exercise training. Both strains have contrasting cardiovascular risk profiles with the SD highly susceptible to development of hypertension (Erdely *et al.* 2003) and the F344 as relatively resistant (Hall *et al.* 1976; Goldstein, 1988). Both strains are also susceptible to age-related renal injury, although the SD progresses more rapidly (Erdely *et al.* 2003; *Lipman et al.* 1996). We sought to investigate if differences in renal hemodynamic responses to exercise between the strains may account for the differences in renal endothelial enzymes with exercise. However, using the radiolabeled microsphere method to measure real-time total RBF during exercise and at rest, we detected similar responses between the strains (Chapter 3). In untrained SED SD and SED F344 rats, RBF significantly fell during acute exercise compared to at rest; however, this response was lost in the chronically TM EX-trained rats for either strain. In addition, resting RBF reduced with chronic TM for both strains. Our findings do align with those by Armstrong and Laughlin who showed that in SD rats chronic exercise reduced the magnitude of the decrease in RBF after acute treadmill-exercise (Armstrong & Laughlin, 1984). Therefore, differences in the renal eNOS and EC SOD response to exercise and susceptibility to IR-induced AKI are not related to differences in handling of RBF. We can speculate that there are differences in intrarenal shear stress patterns which may depend on vessel radius and local viscosity in addition to vessel radius. Given the complexity of the renal circulation, it is also plausible that architectural differences within

the renal vasculature also contribute. This is a particular concern for the SD rat where there is reduced renal eNOS and EC SOD with chronic exercise despite maintained RBF. Perhaps the attenuation of decreased RBF with training is a maladaptive response. We can speculate that systems governing reductions in RBF can also play a role. For example, the renal sympathetic nervous system or hormones such as angiotensin II, endothelin-1, and vasopressin which have been previously reported to dictate exercise-induced falls in RBF (Mueller *et al.* 1998; Stebbins *et al.* 1995, Ahlborg *et al.* 1995; Maeda *et al.* 2004; Stebbins *et al.* 1993).

In taking an evolutionary perspective, it is likely that the role of exercise was to respond to threat. According to Walter Cannon, the fight or flight response describes the increased sympathetic outflow which results in an attempt to prime an animal for preparation of combat (i.e. fight) or for preparation of fleeing as to avoid the combat (i.e. flight). We can speculate that endurance running in hominids evolved as a by-product of enhancing walking capabilities, and was beneficial for effective scavenging as well as for survival tactics. The stress exerted during exercise served benefit for the original hominids; however, in present times, does the risk of exercise serve the same purpose? Thus, the physiological responses we describe herein must take into account the evolutionary perspective of exercise.

This work heavily focuses on impacts of exercise on the kidney but we must also acknowledge other “inactive” areas such as nonworking skeletal muscles and the splanchnic circulation where there is decreased metabolic demand. For example, acetylcholine-induced vasodilation in arterioles of the spinotrapezius, a skeletal muscle that does not exhibit increased blood flow during exercise, has been shown to increase

with training (Lash *et al.* 1997; Xiang *et al.* 2005). In humans, several studies have demonstrated that lower-limb exercise training improved brachial artery flow-mediated dilation, a region that would be considered nonworking during the exercise (Walsh *et al.* 2003; Watts *et al.* 2004). These benefits may be due to the actions of retrograde and oscillatory shear that are increased in the brachial artery as a result of increased resistance in the lower limbs (Simmons *et al.* 2011). Further, some but not all studies also reported improved mesentery vascular reactivity via acetylcholine-induced or flow-mediated vasodilation in mesentery of rats with exercise (Chen *et al.* 2001; Sun *et al.* 1998). Thus, vascular benefits of exercise can extend to regions of decreased metabolic demand. Perhaps there are strain differences in the response to exercise in these areas as well. It is likely that shear stress-independent mechanisms may also contribute to the vascular benefits of exercise.

Studies described herein also suggest that the renal response of exercise is influenced by age. Instead of using an acute model of renal injury as in Chapter 3, we sought to determine the superimposing impact of the aging kidney with exercise since there is NO deficiency and increased oxidative stress in this model as well (Erdely *et al.* 2003; Gomes *et al.* 2009). As described in Chapter 4, using young (16 months) and aged (22-24 months) male F344 rats only, we discovered that 10-12 weeks of TM EX did not alter the age-related renal injury. Rats developed age-dependent renal damage and exercise was not able to reverse it. We also observed an age-dependent loss of the kidney cortex antioxidants EC SOD and CuZn SOD, and age-dependent increase in p22phox which were not influenced by exercise. There were also no significant changes in renal function with exercise in the aged F344 rats. These findings do not

align with Lichtig et al. who demonstrated that 6 weeks of exercise magnified age-associated renal structural injury in old C57BL/6J (Lichtig *et al.* 1987).

From data in Chapters 2-4, it is evident that the F344 background is a strain that is protected against any potential negative influence of exercise on the kidney. Our lab has previously shown that protection against development of renal injury associates with having a maintained NO system which is influenced by genetic background. For example, in the Wistar-Furth rat, resistance to CKD-induced by puromycin administration parallels with preserved NO production (Erdely *et al*. 2004). In contrast, in the SD rat, development of significant renal injury after 11 weeks of 5/6 ablation/infarction is accompanied with falls in renal and total NO production (Erdely et al. 2003). Moreover, age dependent CKD develops slowly in the Munich-Wistar rat and is minimal in WAG/Rij (Baylis and Corman, 1998). Studies in Chapter 5 extend these findings since we found minimal age-related renal injury in the F344XBrown Norway rat that was associated with increased renal NOS protein abundance and no change in renal cortical ADMA levels (Moningka *et al.* 2011). These data are not surprising since it has been shown that the F344xBN rat has fewer glomerular lesions and a greater mean age at which 50% mortality occurs compared to the regular F344 (F344: 103 wks. vs. F344xBN: 145 wks; Lipman *et al.* 1996). Further, we discovered that the tubulointerstitial injury seen with aging in the F344xBN rat is reversed with 6 months of ARB but not ACEI and is not associated with renal NOS. Altogether, our findings in the aging F344XBN confirm that protection against age-related renal injury is associated with a preserved renal NO system. These studies will provide important essential information for aging investigations where various aging models are employed.

Limitations and Future Directions

There are several limitations to the studies presented in this body of work. The majority of our studies used VWR EX which circumvented use of additional stimuli (i.e. electric shock or air jet stress) for motivation to run as seen with TM EX. However, in using VWR we could not control the intensity or adherence of exercise. This limitation was reflected in the high variability of running activity for all rats. Regardless, voluntary running activity was comparable between both strains. TM EX served its purpose of achieving a set exercise intensity of 15 m/min but we cannot rule out the possibility of stress-induced responses since electric shock or jet stress which was applied in our studies. To minimize this, TM EX was conducted with vigilant care to ensure that rats were properly acclimated so that they would not require the use of additional stimuli. In addition, rats were not on a reverse light:dark cycle when trained on the TM which may have confounding effects on their circadian rhythm. We did note that with VWR EX, both strains predominately ran in the dark or during their 'wake' cycle.

As for our microsphere studies, we were limited by the assumption that there was adequate distribution of microspheres upon injection. We assessed this by comparing right and left kidney blood flows and excluding rats with a percent difference greater than ~30. It was evident that greatest variability between left and right kidney blood flows was during the exercise period. It is not certain whether this high variability was reflecting a physiological response of the kidney with exercise, or as a result of inadequate microsphere distribution which can be due to clumping or microsphere entrapment. Despite these limitations, the microsphere technique in determining real-time blood flow during exercise remains very powerful and informative. To date, intrarenal shear stress levels have never been characterized given the complex and

intricate structure of the kidney, and it is not certain if this will ever be achieved. Without technological advancements, there will always be inherent difficulties in determining real-time changes in the kidney during exercise. Nonetheless, these assessments may help uncover factors for why we detected differences in the renal eNOS and EC SOD response to exercise between strains despite their similarities in RBF handling. An indirect approach would be to measure c-Src levels in the kidney since it has been previously shown that this protein tyrosine kinase is a required signaling component of the shear stress response (Davis *et al.* 2003). Where shear is reduced, c-Src levels will also fall. One can then determine if c-Src levels in the kidney are altered with exercise. There are also transcriptional factors that can bind to shear stress response elements located on promoters of specific genes related to shear which can be measured. These include NF kappa B and Egr-1 (Gimbrone *et al.* 2000). There is likely considerable transcriptional regulation of genes involved in the shear stress response.

We must also acknowledge the limitations of basing our interpretations on protein abundance only. Our observations represent a snap shot of what is occurring in the kidney and do not take into time and location. Measurements of enzyme activity could have also strengthened our findings. However, enzyme activity assays are limiting in that they lack the in vivo milieu and provide conditions of optimal enzyme co-factors and substrate supply. Since we used whole tissue homogenates we could not determine the structure or location of the changes seen with exercise. We did conduct immunohistochemical approaches to determine eNOS localization but these were done with 6 weeks of VWR only. In those studies, we also did not assess vessel diameter

changes in response to VWR EX or between strains. Again, the key will be to determine location of change by characterizing intrarenal shear stress patterns.

Another limitation in this study is the correlative and not causative relationship between renal eNOS and EC SOD status and susceptibility to IR-induced AKI. Indeed, we detected strain difference responses in severity of IR-induced AKI but we cannot ascertain if these were directly due to differences in renal eNOS and EC SOD. One approach to address this is to determine impact of exercise and IR injury in either eNOS or EC SOD kidney specific knock-out mice. Increased severity to IR injury with exercise training in either mouse model would confirm our findings and directly implicate the role of either protein.

In hindsight, there are several improvements to our IR studies that could have improved our findings. In our UNX-IR rats, it is uncertain whether conducting the UNX during the IR has any influence. It is possible that clamping of the left the kidney creates hyperfiltration to the contralateral, right kidney, and therefore serves as a potential confounding factor. Secondly, our protein data in the IR studies compared control, contralateral kidneys taken ~24 hours after cessation of VWR versus UNX-IR kidneys taken ~48 hours after cessation of VWR. To circumvent this, future studies should include an additional group of SED and VWR rats who will not have UNX.

In our aging studies, exercise was not efficacious in altering age-related renal injury. However, it is possible that benefits of TM EX were masked since onset of training was at old age. Furthermore, in these studies, as described in Chapter 4, we studied the inbred F344 rat obtained from both the original National Institute of Aging vendor, Harlan, and the current vendor, Taconic Farms. We acknowledged the different

vendors but it is a limitation of our study that we also detected disparities between F344 rats obtained from either vendor (i.e. significant body weight differences between young F344 supplied from Taconic and Harlan).

In this body of work we did not explore sex differences. It is well known that male gender is another risk factor for development of age-associated renal injury. Female SD rats exhibit maintained renal NOS protein abundance with age and are protected against age-dependent kidney damage compared to males (Erdely *et al.* 2003; Baylis 2009). The sexual dimorphism seen with NO deficiency as it relates to aging is presumably due to sex hormones. Few studies have investigated the role of sex differences in impact of exercise on the kidney. It is likely that males will parallel our SD findings in that they will be at increased risk of developing IR-induced AKI.

We must also not exclude the potential influencing role of metabolic pathways. Exercise has numerous metabolic actions including increased insulin sensitivity and decreased plasma triglycerides (Sasaki & Gisele, 2005). We did not consider the roles of angiotensin II and/or vasopressin which are known to influence exercise-induced falls in RBF (McAllister, 1998). However, in our studies, we did not make any assessment of these factors. It is plausible that the marked strain differences in the renal eNOS and EC SOD response to exercise were due to metabolic actions on the endothelium. Indeed, we failed to detect a lack of hemodynamic difference between the strains with exercise. Moreover, although body weight significantly reduced, we found that 3 weeks did not affect renal eNOS and EC SOD in the SD rat. We expected marked changes in renal eNOS and EC SOD since training adaptations were detected (i.e. body weight significantly reduced). Therefore, it is likely that control of renal eNOS and EC SOD is

heavily influenced by systems other than shear stress such as various metabolic signaling pathways.

To study the impact of reduced RBF without influence of the metabolic changes due to exercise, future studies can incorporate the use of the Goldblatt model of renal artery stenosis where there is physical obstruction of the renal artery by clipping. It would be informative to use this model to study the impact of intrarenal eNOS protein abundance without the influence of exericse's metabolic actions.

Several rat studies suggest that the cardiovascular benefit of exercise does not necessarily guarantee renal benefit in the setting of CKD where both cardiovascular and renal complications are present. Bergamaschi et al. reported that 60 days of treadmill exercise did not prevent CKD-induced proteinuria and glomerular slcerosis despite normalization of hypertension (Bergamaschi *et al.* 1997). In contrast, Adams et al. found that voluntary exercise did not ameliorate the hypertension associated with the 5/6 nephrectomy model (Adams *et al*. 2004). These findings will be highly clinically relevant since cardiovascular co-morbidities are high in renal disease patients. To address this, future studies should exploit the differences between the 5/6 ablation and the 5/6 ablation/infarction models of CKD. Both are models of renal mass reduction; however, in the 5/6 ablation/infarction only, there is significant systemic hypertension associated its disease progression (Ibrahim & Hostetter, 1998). It may be the case that exercise has substantial systemic cardiovascular benefits (i.e. reduces the hypertension), and the risk of potential "harm" to the kidney are outweighed. Altogether, these studies would determine whether there is a significant systemic component to the benefit of exercise in a setting where renal injury is present.

Extending our findings in rats to humans also has its limitations. Similar to humans, rats exhibit declines in RBF with exercise; however, the mechanisms involved in sympathetic nervous system activation or VO_2max regulation may uncover species differences. It is also difficult to speculate which rat studied in this body of work is more 'human'. There is considerable genetic impact on susceptibility to renal injury in man so it is likely there are those that will respond like the SD, while others will respond like the F344.

Clinical Perspectives

Renal failure afflicts a growing population in the United States and according to the 2010 U.S. Renal Data System Annual Report these numbers are expected to grow. Benefits of physical activity are critical for this population given the numerous cardiovascular-related benefits. For patients with kidney disease, the National Kidney Foundation recommends to exercise at least three days a week for ~30 minutes a session, and that it must consist of either continuous movement of large muscle groups (i.e. walking, swimming, bicycling, and aerobic dancing) or low-level weight bearing exercise (i.e. high repetition of low weight lifting). They also recommend that participation in any sort of exercise program must consider type, duration, frequency, and intensity of exercise. Indeed, potential risks with exercise should be considered. The data in this body of work are provoking in that we have discovered certain settings where exercise can exacerbate renal injury. Genetic background is clearly a major factor which is highly clinically relevant since there is huge influence of genetics in development of renal injury in humans. The purpose of these studies was not to support discontinuation of exercise prescription for the renal disease population, but to

contribute new knowledge so that maximum benefit is obtained with exercise of optimal modality and intensity.

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BIOGRAPHICAL SKETCH

Natasha Moningka was born in Jakarta, Indonesia in 1983. She and her family emigrated from Indonesia to California where she attended and graduated from Redlands High School in 2002 and the University of California at Irvine in 2006 with a degree in biological sciences. As a result of her undergraduate research work under the mentorship of Dr. Kenneth Baldwin in the Department of Physiology & Biophysics studying responses of skeletal muscle hypertrophy to L-Arginine supplementation, she was a recipient of University of California at Irvine's School of Biological Sciences Excellence in Research. In fall of 2006, Natasha entered the Interdisciplinary Program in Biomedical Sciences at the University of Florida where she joined the laboratory of Dr. Chris Baylis in the Physiology and Functional genomics a year later. As a result of her dissertation work on impact of exercise on the renal nitric oxide and antioxidant systems, she attended and presented at conferences yearly, received the University of Florida Medical Guild Research Incentive Award in 2008, received the Young Investigator Award in 2010 by the Society for Experimental Biology, and published several abstracts and manuscripts. Natasha also serves on the Water & Electrolyte Homeostasis Section Steering and Trainee Advisory committees of the American Physiological Society.

In fall of 2011, she received her Ph.D. from the University of Florida and is currently pursuing her scientific career as a postdoctoral researcher in the laboratory of Dr. Michael Caplan at Yale University where she studies mechanisms of polycystic kidney disease.