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Pharmacological and non-pharmacological approaches to prevent hypertension-induced renal disease in the spontaneously hypertensive rate

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PHARMACOLOGICAL AND NON-PHARMACOLOGICAL APPROACHES TO PREVENT HYPERTENSION-INDUCED RENAL DISEASE IN THE SPONTANEOUSLY HYPERTENSIVE RAT

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DEDICATION

This dissertation is dedicated to my amazing parents, Jeffrey and Lynn Elks. This work is not just a personal achievement, but a testament of their love, encouragement, patience, and support. This work is also dedicated to all lab animals - they are the unsung heroes of biomedical research.

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LIST OF ABBREVIATIONS

SHR	=	spontaneously hypertensive rat
NF-κB	=	nuclear factor-kappa B
AP	=	arterial pressure
HTN	=	hypertension
mmHg	=	millimeters of mercury
PIC	=	proinflammatory cytokine
ROS	=	reactive oxygen species
TNF-α	=	tumor necrosis factor-alpha
IL-1β	=	interleukin-1 beta
IL-6	=	interleukin-6
PDTC	=	pyrrolidine dithiocarbamate
AngII	=	angiotensin II
JAK/STAT	=	Janus kinase/signal transducers and activators of transcription
RAS	=	renin angiotensin system
VCAM1	=	vascular cell adhesion molecule 1
ICAM1	=	intercellular adhesion molecule 1
DNA	=	deoxyribonucleic acid
NAD(P)H	=	nicotinamide adenine dinucleotide phosphate
NO	=	nitric oxide
SOD	=	superoxide dismutase
Cr	=	creatinine
BUN	=	blood urea nitrogen
GFR	=	glomerular filtration rate

RBF	=	renal blood flow
AT-1	=	angiotensin II type 1
ACE	=	angiotensin converting enzyme
ARB	=	angiotensin receptor blocker
CCB	=	calcium channel blocker
BP	=	blood pressure
ExT	=	exercise training
DASH	=	Dietary Approaches to Stop Hypertension
BB	=	blueberries
ORAC	=	oxygen radical absorbance capacity
ATP	=	adenosine triphosphate
PAH	=	para-aminohippurate
CMH	=	1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine
CPH	=	1-hydroxy-3-carboxypyrrolidine
PPH	=	1-hydroxy-4-phosphono-oxy-2, 2, 6, 6-tetramethylpiperidine
DF	=	defferoxamine
DETC	=	diethyldithiocarbamate
KHB	=	Krebs-Hepes buffer
PEG-SOD	=	polyethylene glycol-conjugated superoxide dismutase
WKY	=	Wistar-Kyoto
Hct	=	hematocrit
ELISA	=	enzyme-linked immunosorbent assay
EGTA	=	ethylene glycol tetraacetic acid
MOPS	=	3-(N-morpholino) propanesulfonic acid

BSA	=	bovine serum albumin
VDAC	=	voltage dependent anion channel
IκBα	=	inhibitor of kappa B alpha
GAPDH	=	glyceraldehyde 3-phosphate dehydrogenase
EMSA	=	electrophoretic mobility shift assay
RNA	=	ribonucleic acid
RT-PCR	=	reverse transcriptase-polymerase chain reaction
NOX	=	NADPH oxidase
EPR	=	electron paramagnetic resonance
SEM	=	standard error of the mean
ANOVA	=	analysis of variance
MAP	=	mean arterial pressure
SBP	=	systolic blood pressure
UA	=	urine albumin
OD	=	optical density
RVR	=	renal vascular resistance
BW	=	body weight
PAS	=	periodic acid-Schiff reagent
NT	=	nitrotyrosine
GPx	=	glutathione peroxidase
GSH	=	reduced glutathione
GSSG	=	oxidized glutathione
eNOS	=	endothelial nitric oxide synthase
iNOS	=	inducible nitric oxide synthase

RNS	=	reactive nitrogen species
SHRSP	=	spontaneously hypertensive stroke-prone rat
JSP	=	Japanese stroke prone
SIRT1	=	sirtuin 1
JNK	=	c-Jun N-terminal kinase
KW	=	kidney weight

ABSTRACT

Hypertension affects 50 million Americans and remains the second leading cause of renal failure in the United States. Current pharmacological and non-pharmacological approaches to treat hypertension have proven effective, but the complexities of the disease and its renal effects warrant the need for new treatments. The hypothesis of this dissertation was that pharmacological or non-pharmacological approaches to reducing inflammation and oxidative stress would prevent hypertension-induced renal injury in the spontaneously hypertensive rat (SHR).

In the first study, we blocked the inflammatory transcription factor, nuclear factor-kappa B (NF-κB), with pyrrolidine dithiocarbamate in the SHR kidney. In treated SHR, blood pressure decreased, renal hemodynamics were preserved, and oxidative stress and inflammation were attenuated at both the cytosolic and mitochondrial levels; suggesting a role for NF-κB in potentiating hypertension-induced renal injury.

In the second study, we examined the effects of aerobic exercise training on renal oxidative stress and inflammation. Exercised SHR exhibited normalized blood pressure and renal hemodynamics. These effects were attributed to lower NF-κB activity and decreased oxidative stress in the SHR kidney. In the third and fourth studies, we examined the effects of diet modification by use of blueberry-enriched diets, since blueberries have one of the highest antioxidant capacities of any fruit or vegetable tested to date.

In the third study, we fed stroke-prone SHR high salt and a blueberry-enriched diet for 2 days, 6 weeks, or 12 weeks, and examined renal parameters. The SHR fed the blueberry diet for the 6- or 12-week periods demonstrated lower oxidative stress, lower blood pressure, and preservation of renal hemodynamics. These effects were likely due to a hormetic effect of the

blueberries themselves, since rats fed blueberries for 2 days demonstrated higher oxidative stress. In the final study, we added blueberries to a stroke-permissive diet, which accelerates renal damage in SHR. Rats were fed diets for 10 weeks. Rats fed the control diet had severe hypertension, severe oxidative stress, and severe inflammation as evidenced by NF-κB activation, and exhibited signs of renal failure. Rats fed the blueberry supplemented diet exhibited decreases in blood pressure, oxidative stress, and inflammation, and also had preserved renal structure and function.

CHAPTER 1

REVIEW OF LITERATURE

Arterial Pressure

Blood pressure is the force exerted by circulating blood on the walls of the arteries and veins. Venous pressure is commonly very low and does not significantly factor into the determination of blood pressure; therefore, the term 'arterial pressure (AP)' is often used. With each heartbeat, the AP varies between systolic and diastolic pressures. Systolic pressure is the peak pressure in the arteries, which occurs near the end of the cardiac cycle when the ventricles are contracting to eject blood. Diastolic pressure is the minimum pressure in the arteries, which occurs near the beginning of the cardiac cycle when the ventricles are filled with blood.

Maintenance of AP at a homeostatic level - one that allows for adequate blood supply to the body tissues - is critical to the survival of mammals. Several mechanisms exist that maintain homeostatic AP, even in situations that are stressful to the body, including: alterations in salt and water intake, and hemorrhage [1]. Some mechanisms function rapidly and are responsible for minute-to-minute regulation of AP, while others are responsible for regulating AP over longer time periods. Other mechanisms are involved in both short- and long-term regulation of AP. The involvement of hormonal and neural factors in determining and maintaining AP is well-established; however, the kidney's dominance of the long-term regulation of AP is clear. Guyton and colleagues first proposed the theory that AP is sustained at a 'set-point' required by the kidneys to maintain a balance between intake and excretion of sodium and water [1].

Hypertension

Overview and Prevalence

Hypertension (HTN), or persistently high AP, is the most common primary diagnosis in the United States [2]. Current estimates indicate that approximately 73.6 million (one in three) Americans presently suffer from HTN, and that 22% of these individuals are undiagnosed [2]. Hypertension is often called the "silent killer", due to the considerable damage it inflicts while the patient remains asymptomatic. The devastating effects of HTN are not limited to a single tissue; rather, they adversely affect most organs and organ systems [2]. Hypertension is a major contributing factor to atherosclerosis, which causes an increase in the relative workload for the heart, which must pump blood under greater peripheral resistance. Over time, the greater workload may result in myocardial infarction and/or heart failure. The brain and its delicate arteries can also be affected by HTN. Over time, these vessels may rupture, resulting in brain hemorrhage or cerebrovascular accident (stroke). Finally, and arguably most importantly, HTN can result in thickening or disease of the arteries and arterioles of the kidneys, thereby decreasing glomerular filtration rate and renal blood flow. In response to these alterations, the kidneys may secrete renin, which elevates AP further and perpetuates this positive feedback cycle.

Risk Factors for Hypertension

The risk factors for HTN can be classified into two categories: non-controllable and controllable. Non-controllable risk factors for HTN include age, gender, genetic predisposition, and ethnicity. Controllable risk factors are those that can be altered, such as high salt or high fat diet, excess body weight, smoking, and lack of exercise. In the United States, African-Americans have a higher prevalence of HTN than do non-Hispanic whites [3, 4]. The morbidity associated with HTN is also more severe among African-Americans and is thought to develop

earlier in life [5]. Age is another important factor in the development of HTN. Longitudinal research studies have found increases in AP with age, with diastolic pressure peaking around age 50 and systolic pressure rising throughout life [6-8]. Recently, an increased prevalence of HTN has been noted among adolescents as a result of obesity, diet, and a sedentary lifestyle [9]. If these cases remain uncontrolled, morbidity and mortality may result for these individuals at significantly earlier ages. Gender also influences the onset of hypertension [9]. Hypertension is less prevalent in women in early adulthood; however, the incidence of HTN increases rapidly in women above the age of 40. The prevalence of hypertension in females equals or possibly exceeds that in men by age 50 [9].

Classification

Hypertension can be classified as primary (essential) or secondary [4]. The term essential hypertension encompasses about 90-95% of all diagnosed cases, and is clinically defined as an elevation in AP of unknown etiology [4]. Secondary hypertension represents about 5-10 % of al1 diagnosed cases and can generally be reversed with correction of the underlying cause [10]. Despite the extensive research efforts of recent years and the current knowledge of the physiology of AP regulation, the basis of essential HTN in most patients remains elusive. Patients are categorized, based on systolic and diastolic AP readings, as either normotensive, prehypertensive, or hypertensive [4, 9]. The National Heart, Lung and Blood Institute further divides the hypertension classification into two stages (Table 1.1), with stage two being the more severe stage.

Table 1.1 Classification of hypertension, based on the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure [2].

Category	Systolic Blood Pressure (mmHg)		Diastolic Blood Pressure (mmHg)
Normal	<120	and	<80
Prehypertensive	120-139	or	80-89
Hypertension (Stage 1)	140-159	or	90-99
Hypertension (Stage 2)	≥160	or	≥100

Kidney Redox Mechanisms in Hypertension

Hypertension-induced kidney disease is a significant cause of morbidity and mortality in hypertensive patients [11]. Current anti-hypertensive treatments are mostly effective in reducing the severity of hypertensive renal disease; however, the progressive clinical course of the disease underscores the need for additional novel therapies. The progression of hypertensive kidney involves increased pro-inflammatory cytokine (PIC) and reactive oxygen species (ROS) production, and on nuclear factor-kappa B (NF-κB) activation [12, 13].

Renal Inflammation and Hypertension

Increasing evidence supports a role for inflammatory molecules in the pathogenesis of hypertension [14]. In the kidney, tubulointerstitial inflammation is accompanied by activation of NF-κB [15, 16] which is the general transcriptional factor for many proinflammatory cytokines, chemokines, and adhesion molecules. A role for renal inflammation in the pathogenesis of hypertension is demonstrated by a number of animal studies that have shown attenuation of hypertension with anti-inflammatory compounds, including mycophenolate mofetil and NF-kappa B inhibitors [17-20]. We recently reported decreased plasma concentrations and renal gene and protein expression of the proinflammatory cytokines tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) in spontaneously hypertensive rats (SHR) treated with pyrrolidine dithiocarbamate (PDTC) [21]. Those animals also had decreased

glomerular desmin expression when compared to untreated SHR, which is indicative of less glomerular damage in these animals, presumably due to a decrease in the inflammatory condition.

Vascular inflammation also affects the kidney during hypertension. A major factor underlying this type of inflammation is modulation of proinflammatory gene expression via redox-sensitive transcription factors [20, 22]. Angiotensin II (AngII) has an important role in modulating expression of pro-inflammatory molecules in the vasculature [14]. AngII induced the release of IL-6, which caused the recruitment of inflammatory cells from human vascular smooth muscle cells into the vessel media; this release required the production of ROS and activation of NF-κB [23]. Also involved in pro-inflammatory gene transcription are the Janus kinase/signal transducers and activators of transcription factors (JAK/STAT) pathways. These pathways can be activated by exogenous hydrogen peroxide, other ROS, and AngII [24, 25].

A number of the pathways responsible for adhesion molecule expression in the kidney are also redox-sensitive [26]. In the double transgenic rat, a model of overactive renin-angiotensin system (RAS) and oxidative stress, vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) expression are elevated in the small renal vessels [27]. Further, ICAM-1, VCAM-1, and monocyte chemoattractant protein-1 expression levels were all reduced in a salt-sensitive hypertension rat model after blockade of TNF-α [28]. AngII-infused hypertensive rats also demonstrate increased VCAM-1 expression due to NF-κB-mediated transcriptional events [29].

Renal Oxidative Stress and Hypertension

In normal conditions, mammalian cells reduce molecular oxygen to water during aerobic respiration. During this process, ROS are generated that include superoxide, hydroxyl radicals,

and hydrogen peroxide; these highly reactive species may interact with proteins, lipids, and deoxyribonucleic acids (DNA) [30, 31]. Normally, the *in vivo* antioxidant defense is sufficient to metabolize these ROS; however, in conditions of persistent oxidative stress, antioxidant molecules and enzymes can be depleted and inactivated, thereby impairing the overall antioxidant defense system [31]. Excessive ROS production causes oxidative damage and is associated with hypertension [32, 33] and other diseases. The major producers of these ROS include plasma membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and mitochondria [21].

ROS also have an important role in the inactivation of nitric oxide (NO) [34, 35]. When oxidative stress occurs, endothelial dysfunction and reduced NO bioavailability will result, usually from superoxide binding to NO to form peroxynitrite [34, 36]. This reduced NO bioavailability leads to several processes that contribute to hypertension: inflammation with increased proinflammatory cytokine production, lipid peroxidation, and increased vascular contractility [37]. We have demonstrated increases in production rates of superoxide, total ROS, and peroxynitrite in the kidneys of spontaneously hypertensive rats; these increases were attenuated with the administration of the antioxidant and NF-κB inhibitor, PDTC [21].

Oxidative stress contributes to hypertension, endothelial/vascular dysfunction, and brain disorders in animals with chronic kidney diseases. This is partly due to the up regulation of NADPH oxidase and the down-regulation of superoxide dismutase (SOD) [15, 22, 30]. Several studies, including one from our lab, support the hypothesis that uremia in renal disease is associated strongly with oxidative stress. We demonstrated increased plasma creatinine (Cr) and blood urea nitrogen (BUN) levels, along with decreases in glomerular filtration rate (GFR) and renal blood flow (RBF), in SHR (a model of increased oxidative stress) [21]. Oxidative stress is

also known to exacerbate renal injury. In patients with end stage renal disease, hemodialysis or peritoneal dialysis treatment for longer than two years can lead to further reduction of antioxidant levels and increased oxidant levels, thereby perpetuating the oxidative stress seen in these patients. Further, there is a greatly decreased potential for scavenging of oxygen radicals after about seven years of hemodialysis in patients [38]. Therefore, the treatments for renal failure can also aggravate the oxidative stress that initially contributed to the disorder.

The Relationship Between Oxidative Stress and Inflammation in the Kidney

The relationship between inflammation and oxidative stress is notable. Oxidative stress can activate NF-κB [31, 39] and activator protein-1 [31], stimulating production of chemokines, cytokines, and adhesion molecules, as well as activation and proliferation of lymphocytes. Immune cell activation, adhesion, and infiltration result from these events. Conversely, inflammation can result in oxidative stress, since ROS production is an inherent property of activated immune cells. Renal inflammation is thought to be a key mediator in the development and progression of hypertension, and compelling evidence suggests that ROS overproduction and NF-κB activation promote glomerular and tubulointerstitial inflammation in rat models of hypertension [40, 41]. Blockade of NF-κB or of ROS has demonstrated both anti-hypertensive and anti-inflammatory effects in rats [17, 42, 43]. Further, immune cells infiltrating the kidney have been shown to produce ROS in hypertensive animals and humans [30]. Thus, oxidative stress and inflammation seem to be involved in a vicious, self-perpetuating positive feedback cycle.

Role of Kidney RAS in Inflammation and Oxidative Stress

Angiotensin II stimulates salt retention directly in the proximal tubule and indirectly via aldosterone in the distal tubule, and also causes vasoconstriction [44]. In addition to these

common physiological effects, AngII also activates the angiotensin II type 1 (AT-1) receptor, resulting in renal ROS generation via the up regulation of NADPH oxidases [44-46]. The ROS produced by increased NADPH oxidase expression and activity can activate NF-κB [45]. Angiotensin II can also directly activate NF-κB in various renal cells, including mesangial and endothelial cells, which leads to inflammation in these tissues [47, 48]. Inflammation can also increase the expression of several components of renal RAS, including AngII, renin, and AT-1 receptor [49, 50]. A variety of animal studies have shown marked up regulations of RAS components in the kidneys of hypertensive animals [49, 51-53]. In renal damage associated with activated RAS, increased renal NF-κB activity was diminished by angiotensin converting enzyme (ACE) inhibition [54-56]. We and others have demonstrated that the blockade of NF-κB can improve renal tissue damage and renal hemodynamics [21, 57]. *In vivo*, systemic infusion of AngII into normal rats increases NF-κB activity in the vasculature and the kidney [58]. Therefore, the RAS and NF-κB appear to be involved in another vicious cycle that contributes to hypertension, inflammation, and renal damage.

Pharmacological Treatment of Hypertension

Substantial clinical trial outcome data exist to support the use of several classes of pharmaceuticals to reduce hypertension and its associated complications [59-63]. Commonly used classes of drugs include: thiazide-type diuretics, ACE inhibitors, angiotensin receptor blockers (ARBs), calcium channel blockers (CCBs), and beta blockers [9]. Thiazide-type diuretics have long been the basis of antihypertensive therapy. In most clinical trials, including the Antihypertensive and Lipid Lowering Treatment to Prevent Heart Attack Trial (ALLHAT) [64], diuretics have gone unsurpassed in preventing the cardiovascular complications of

hypertension, and can also enhance the antihypertensive efficacy of combination drug regimens, and are more affordable than other antihypertensive agents [64].

In the last decade, several large trials comparing the newer antihypertensive agents, including CCBs, ACE inhibitors, and an ARB, with commonly prescribed diuretics and/or beta blockers have been completed [60, 61, 63, 64]. Most of these studies demonstrated that these newer antihypertensives were neither superior nor inferior to the older drugs in terms of efficacy. However, more than two-thirds of hypertensive individuals will require two or more antihypertensive agents selected from different drug classes for adequate treatment of hypertension [60, 61, 65].

Non-Pharmacological Treatment of Hypertension

Extensive published evidence supports the concept that non-pharmacological interventions (also referred to as lifestyle modifications), can substantially reduce blood pressure (BP) in individuals with established hypertension or with prehypertension. Epidemiological data has implicated several dietary and other lifestyle-related factors in contributing to the development of hypertension. These factors include: habitual high sodium intake, low potassium intake, decreased physical activity, high body mass index, and excess alcohol intake [66, 67].

Aerobic Exercise Training

A sedentary lifestyle is a well-established independent risk factor for cardiovascular diseases. Epidemiological evidence suggests that a greater level of physical fitness is associated with lower BP and a reduction in morbidity and mortality from cardiovascular events [68]. Regular physical activity can reduce the risk of developing hypertension by 30-50 % [68]. Further, regular moderate physical activity has been shown to decrease systolic BP by 6-10 millimeters of mercury (mmHg) and diastolic BP by 4-8 mmHg in patients with essential

hypertension [69]. In a meta-analysis of 54 randomized controlled trials with a sample size of approximately 2400, aerobic exercise training (ExT) was associated with a statistically significant reduction in BP [70]. This BP reduction was similar in normotensive and hypertensive participants. Perhaps most importantly, mean BP reduction was not associated with changes in body weight, and BP was reduced significantly, even in participants that did not lose weight. The mechanisms surrounding BP reduction with aerobic exercise training remain unclear; however, a reduction in vascular resistance is thought to be a major effect. Decreased oxidative stress is also thought to play a role in BP reduction in response to ExT [69].

Six weeks of moderate intensity ExT was found to preserve renal function (as indicated by increased natriuresis and decreased proteinuria) by decreasing oxidative stress and improving antioxidant defense in the aged rat kidney [71]. A recent study in obese rats found that five weeks of ExT prevented albuminuria and oxidative stress and decreased nuclear translocation of NF-κB in the kidney [72]. Interestingly, ExT can also improve oxidative stress from renal injury prior to the renal injury occurring. Normotensive rats were subjected to ExT for eight weeks and then underwent 5/6 nephrectomy to induce chronic kidney disease. When compared to nephrectomized sedentary rats, the nephrectomized rats subjected to ExT had decreased superoxide production and oxidative insult but no significant improvement in renal function [73]. Further, basal urinary nitrate/nitrite excretion increases with increasing levels of physical activity and these levels are also significantly increased after ExT in patients with coronary artery disease [74].

Diet Modification

In addition to weight loss and regular physical activity, reduction of sodium intake and consumption of a diet rich in low fat dairy products, fruits, and vegetables (and therefore high in

vitamins, minerals, and antioxidants) have been shown to improve BP [75]. The most recent guidelines set forth by the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure recommend the adoption of this type of diet [9, 75], which was formulated for the Dietary Approaches to Stop Hypertension (DASH) study.

The DASH study tested the effects of an overall healthy diet on BP in participants with prehypertension or stage I hypertension [67]. A total of 459 adults with BP <160/80–95 mmHg were randomly assigned for 8 weeks to one of the following diet groups: 1) control diet = low in fruits, vegetables, dairy products with a fat content typical of the average American diet, and with potassium, magnesium, and calcium levels close to the 25th percentile of consumption; 2) fruit and vegetable diet = potassium and magnesium at 75th percentile and high in fiber; or 3) combination diet = rich in fruits, vegetables, and low-fat dairy, with reduced saturated fat, total fat, and cholesterol, and potassium, magnesium, and calcium at the 75th percentile of consumption [67, 75]. Study diets each had a sodium content of approximately 3 grams per day. Interestingly, the combination diet group experienced the most significant decrease in BP; this reduction began during the second week of the diet. Further, the combination diet was found to be as effective in reducing BP as some drug monotherapies. It was therefore concluded that the DASH combination diet might be an effective alternative to pharmacotherapy in patients with stage I hypertension and may provide an opportunity to those patients to delay the start of an antihypertensive drug regimen [67, 75].

The Spontaneously Hypertensive Rat

The spontaneously hypertensive rat (SHR) is a rat strain developed by Okamoto and Aoki in the 1960s by selectively breeding male rats from the Wistar-Kyoto (WKY) strain with BP persistently in the range of 150 – 175 mmHg with WKY females with BP of 130-140 mmHg

[76]. Selective breeding continued until a strain was developed where males develop chronic essential hypertension beginning at approximately 8 weeks of age, with systolic BP often exceeding 200 mmHg [76]. This genetically hypertensive strain is also known to exhibit increased sympathetic nervous system activity, increased RAS activity, and increased oxidative stress [77]. The SHR is a widely used model of human hypertension because it develops many features of hypertensive end-organ damage, including cardiac hypertrophy, cardiac failure, and renal dysfunction [77]. Because the SHR was developed from the WKY rat, the WKY is often used as the normotensive control for this strain.

Blueberries (*Vaccinium* Species)

Constituents and Bioactive Components

North American blueberry (BB) species include highbush blueberry (*V. corymbosum*) and lowbush blueberry (*V. angustifolium*). Highbush BB are cultivated as unique varieties, while lowbush BB grow in the wild. The antioxidant capacity of BB is among the highest of fruits and vegetables [78, 79]. Although BB are rich in vitamins and minerals [80], the contribution of these micronutrients to the antioxidant capacity of BB is minimal [81]. Blueberries contain high levels of flavonoids and polyphenols [81], and are especially high in anthocyanidins. Six anthocyanidins are commonly found in nature, and BB contain five of these anthocyanidins [82]. Malvidin, delphinidin, cyanidin, petunidin, and peonidin are found in all *Vaccinium* species, and are usually found in the form of anthocyanidin glycosides, which are referred to as anthocyanins (from the Greek words “anthos” = flower and “kyanos” = blue) and are responsible for giving BB their unique coloring [82]. Anthocyanins play a vital role in the skin of the blueberry by protecting the fruit’s flesh from overexposure to ultraviolet light.

Significant differences exist in both the types of anthocyanins and total anthocyanin content among *Vaccinium* subspecies [83]. North American wild lowbush BB have higher total anthocyanin levels per 100g of fresh weight than do highbush BB; this is because the majority of anthocyanins are present in the skin of the berry and lowbush BB have a greater amount of surface area per volume of fruit [83]. However, highbush BB are generally the only variety of blueberry available as a whole, relatively unprocessed fruit (either fresh or frozen) in grocery stores. Lowbush BB are used predominantly in processed foods and in flavored yogurt [84, 85]. Processing significantly affects the antioxidant capacity of BB and blueberry products [84, 85]. Although freezing is a form of processing and does result in loss of antioxidant capacity, frozen BB and fresh BB purees retained higher levels of unoxidized anthocyanins than all other forms of processed BB and BB products measured in a study by Kalt and colleagues [85].

Blueberries and Chronic Disease

Many studies have demonstrated the anti-hypertensive benefits associated with consuming diets high in fruits and vegetables; yet there have been very few studies examining the relationship between BB consumption and hypertension, and no studies examining the effects of BB on renal function. However, many recent studies have demonstrated the benefits of BB consumption on brain function [86-90]. A study by Joseph and colleagues examined whether foods with high antioxidant activity, including BB, could reverse age-related declines in neural and behavioral functions. Forty 19-month old male rats were assigned to one of four treatment groups: control, 1.48% strawberry, 0.91% spinach, or 1.86% BB [89]. The percentages of each of these foods in the diets were based on oxygen radical absorbance capacity (ORAC), to ensure that each diet provided an equivalent amount of antioxidants. Diets were fed to rats for eight weeks before neural and psychomotor behavior testing. All foods tested prevented age-related

neuronal and behavioral dysfunction, with BB supplementation showing the greatest effect on reversing the deleterious effects of aging on calcium homeostasis. Further, the BB-fed rats were the only animals that exhibited reversals in motor behavioral deficits [89]. The feeding of a 14.3% BB diet to male Long-Evans rats for six weeks before carotid artery ligation (to induce stroke) was found to be protective against ischemia-induced hippocampal injury [90]. One week after carotid ligation, rats on the BB diet had only a 17% loss of neurons to the ischemic hippocampus compared to a 40% loss in the control group, suggesting that blueberries can protect against ischemic brain injury [90].

One recent study by Ahmet and colleagues examined the effects of BB consumption on ischemic injury in the heart [91]. Fischer rats were fed a control diet or a BB-enriched diet for 3 months. Mitochondrial permeability transition of cardiomyocytes was assessed in seven rats from each group after the 3-month feeding period, and those animals fed a BB-enriched diet demonstrated a 24% increase in mitochondrial permeability transition threshold, indicating an improvement in mitochondrial integrity with BB feeding. Animals from each group were also subjected to myocardial ischemia by coronary artery ligation, and resulting infarct size was 22% less in rats fed the BB-enriched diet, suggesting an overall cardioprotective effect of blueberries [91].

The only study to examine the effects of blueberry consumption on blood pressure was conducted by Shaughnessy and colleagues in the stroke-prone spontaneously hypertensive rat [92]. Stroke-prone SHR and normotensive control rats were fed a control diet or a 3% BB diet for 8 weeks. Systolic blood pressure was 19% lower after 4 weeks in SHR fed the BB diet, and was 30% lower after 8 weeks. Proteinuria and kidney nitrites were also lower in these animals,

suggesting that BB may prevent oxidative damage in the kidney [92]. However, no detailed measurements of renal structure or function were performed in that study.

Statement of the Problem and Specific Aims

Increased oxidative stress and inflammation perpetuate a vicious positive feedback cycle that can exacerbate the hypertensive condition and, if left unchecked, can lead to damage of organs such as the kidney. The redox-responsive transcription factor, NF-κB has been shown to contribute to the deleterious effects of reactive species and inflammatory molecules on kidney structure and function. Although obvious putative roles exist for oxidative stress and NF-κB in the development and progression of hypertension-induced renal disease, the exact signaling mechanisms that perpetuate their effects on the hypertensive kidney remain unclear. The overall hypothesis of this dissertation was that pharmacological or non-pharmacological approaches to reducing both inflammation and oxidative stress would prevent or delay hypertension-induced renal injury in the spontaneously hypertensive rat (SHR), a model for human essential hypertension. We performed a series of *in vivo* experiments, integrated with various physiological and molecular techniques, to fulfill the following aims:

Aim 1: Determine the benefits of pharmacological NF-κB blockade on hypertension-induced renal injury in SHR.

Aim 2: Determine the benefits of exercise training as a non-pharmacological modality for prevention or treatment of hypertension-induced renal injury in SHR.

Aim 3: Examine the short-term and long-term effects of a blueberry-enriched diet on hypertension induced renal injury in stroke-prone SHR.

Aim 4: Examine the effects of a blueberry-enriched stroke-permissive diet on hypertension and hypertension-induced renal injury in salt-loaded stroke-prone SHR.

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CHAPTER 2

CHRONIC NF-κB BLOCKADE REDUCES CYTOSOLIC AND MITOCHONDRIAL OXIDATIVE STRESS AND ATTENUATES RENAL INJURY AND HYPERTENSION IN SHR*

Introduction

Hypertension-induced kidney disease is a significant cause of morbidity and mortality in hypertensive patients [93]. Current anti-hypertensive treatments are mostly effective in reducing the severity of hypertensive renal disease; however, the progressive clinical course of the disease underscores the need for additional novel therapies. The progression of hypertensive kidney disease depends not only on neurohormones, such as norepinephrine and aldosterone, but also on increased proinflammatory cytokine (PIC) and reactive oxygen species (ROS) production, and on nuclear factor-kappa B (NF-κB) activation [94, 95]. Increased production of ROS, which include superoxide and hydrogen peroxide, is a particularly detrimental aspect of renal disease progression [95]. The major producers of these ROS include plasma membrane-bound NAD(P)H oxidases and mitochondria.

Mitochondria are critical modulators of ATP generation and redox-dependent intracellular signaling. The mitochondrial respiratory chain continuously releases ROS during oxidative phosphorylation. Approximately 90% of the cellular oxidative burden is attributed to mitochondrial ROS, thus signifying the role of mitochondria in cellular ROS production [96]. In normal physiological conditions, small amounts of ROS are needed for critical cellular processes; however, excessive ROS production causes oxidative damage and is associated with hypertension [97, 98] and other diseases. The contributions of ROS to the regulation of

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intracellular signaling pathways, including NF-κB activation, are already known. Excess ROS activate the redox-sensitive transcription factor NF-κB, causing increases in its activity and expression [99, 100]. Increased activity and expression of NF-κB induces gene transcription for PIC, such as tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), and IL-6, to increase their production [99, 101]. Increased levels of PIC, along with adhesion molecules, lead to macrophage infiltration of the tubulointerstitium and inflammation of renal tissue [102, 103]. However, the roles of NF-κB and of ROS in modulating renal function and tissue injury in hypertensive renal damage have not yet been examined.

Evidence from our laboratory indicates that peripheral TNF-α administration increases ROS production in rat myocardial tissue and mitochondria [104]. Findings from other labs also indicate that TNF-α augments ROS production in liver mitochondria and endothelial cells [105, 106]. IL-6-dependent ROS production has been noted in fibroblasts and in endothelial cells [107, 108]; and fibroblasts have previously been shown to release ROS in response to IL-1β and TNF-α [109]. Taken together, these data support a role for NF-κB-regulated PIC in cytosolic and mitochondrial ROS production in a variety of tissues. Therefore, it is plausible to suggest that NF-κB blockade (and, therefore, blockade of PIC gene transcription) may improve the redox status of hypertensive renal cortical tissue and mitochondria.

Renal inflammation is thought to be a key mediator in the development and progression of hypertension, and compelling evidence suggests that ROS overproduction and NF-κB activation promote glomerular and tubulointerstitial inflammation in rat models of hypertension [110, 111]. Further, blockade of NF-κB or of ROS has demonstrated both anti-hypertensive and anti-inflammatory effects in rats [112-114]. However, the effects of NF-κB blockade on mitochondrial ROS and the roles of these ROS in modulating renal function and tissue injury in

hypertension are unknown. The aim of this study was to examine the effects of long-term NF-κB blockade on cortical cytosolic and mitochondrial ROS production and on renal function during hypertension, and to investigate the contributions of these ROS to hypertensive renal injury. We hypothesized that long-term NF-κB blockade with pyrrolidine dithiocarbamate (PDTC) would decrease both cytosolic and mitochondrial ROS production in the kidney cortex, thereby protecting the kidneys from damage, and resulting in improved renal function parameters in spontaneously hypertensive rats (SHR).

Materials and Methods

All experimental procedures with animals were approved by the Louisiana State University Institutional Animal Care and Use Committee, and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Chemicals and Drugs

Inulin (polyfructosan-S; Inutest) was obtained from Fresenius-Kabi (Graz, Austria) and para-aminohippurate (PAH) was obtained from Merck, Sharp & Dohme (West Point, PA). The spin probes 1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine (CMH), 1-hydroxy-3-carboxypyrrolidine (CPH), and 1-hydroxy-4-phosphono-oxy-2, 2, 6, 6-tetramethylpiperidine (PPH); the metal chelators defferoxamine (DF) and diethyldithiocarbamate (DETC); and Krebs-HEPES buffer (KHB) were obtained from Noxygen Science Transfer and Diagnostics (Elzach, Germany). Polyethylene glycol-conjugated superoxide dismutase (PEG-SOD), thiobutabarbital (Inactin), and pyrrolidine dithiocarbamate (PDTC) were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals and reagents used were of analytical grade and were purchased from Sigma-Aldrich unless otherwise specified.

Experimental Protocol

Eight-week old male Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR), obtained from Harlan (Indianapolis, IN), with initial body weights between 200-250 grams, were used in this study. Animals were housed in temperature- (23 ± 2°C) and light-controlled (12-hour light/dark cycle) animal quarters. Four groups of 6 rats each were used: WKY, WKY+PDTC, SHR, and SHR+PDTC. All animals were allowed *ad libitum* access to standard rodent chow (LabDiet; Purina Mills Inc., St. Louis, MO). Control animals were given access to tap water *ad libitum*; PDTC-treated rats were allowed *ad libitum* access to tap water with PDTC added. Beginning at 8 weeks of age, the appropriate groups of rats were treated with PDTC dissolved daily in drinking water for 15 weeks. At 8 weeks of age, SHR are still normotensive [115], therefore, we initiated treatment for all animals at this age. For the first week of the study, PDTC was added to drinking water at an initial concentration of 50 mg/kg; the concentration was increased by 25 mg/kg daily until the final concentration of 150 mg/kg was attained; this was done to allow for adaptation to changes in taste. At the end of week 13, rats were placed into metabolic cages for a one-week acclimatization period. Immediately following acclimatization, daily urine output was measured for one week. Animals were sacrificed at the end of week 15, after completion of renal clearance experiments. Plasma and kidney tissues were collected for later analyses.

Blood Pressure Measurement

Blood pressures for all animals were measured by the tail-cuff method at baseline and every third week using a Coda 6 Blood Pressure System (Kent Scientific, Torrington, CT) for the duration of the study as previously described [116]. Animals were habituated to the blood pressure system for 3 days prior to starting the experiment. Rats underwent two daily cycles of

20 measurements each for a minimum of 3 days per week. Body temperatures were monitored for the duration of each blood pressure measurement.

Renal Clearance Experiments

At the end of 15 weeks' treatment, acute clearance experiments were performed to determine renal function in anesthetized (thiobutabarbital, 100 mg/kg; intraperitoneally) rats, as described previously [117]. In brief, the right inguinal area was shaved, a small (< 2 cm) incision made, and femoral vessels isolated. The right femoral artery was cannulated with heparin-primed (100 U/ml) polyethylene tubing (PE-50) and then connected to a pressure transducer (PowerLab data acquisition system; ADInstruments, Colorado Springs, CO) for continuous measurement of arterial pressure. The right femoral vein was catheterized with heparin-primed PE-50 tubing for infusion of various solutions at a rate of 20 μL/min [117, 118]. An isotonic saline solution containing 6% albumin (EMD Chemicals, Gibbstown, NJ) was infused during surgery. After surgery, the infusion fluid was changed to isotonic saline containing 2% bovine serum albumin (BSA), 7.5% inulin (Inutest), and 1.5% PAH. The bladder was catheterized with a PE-90 tube (with one end flanged) via a suprapubic incision for gravimetric urine collection. After a 15- to 20-minute stabilization period, a 30-minute clearance period was conducted to assess values of renal hemodynamic parameters. An arterial blood sample was collected at the end of the 30-minute clearance collection period for measurement of plasma inulin and PAH concentrations. Plasma inulin and PAH concentrations were measured colorimetrically to determine glomerular filtration rate (GFR) and renal plasma flow (RPF), respectively. Renal blood flow (RBF) was calculated from RPF and hematocrit (Hct) using the standard formula: $RBF = RPF \div (1-Hct)$.

Biochemical Assays for Urine and Plasma

Albumin in urine was quantified with a Nephrat II ELISA kit (Exocell, Philadelphia, PA) and TNF-α, IL-6, and IL-1β were quantified in plasma samples with ELISA kits from Biosource/Invitrogen (Carlsbad, CA). Creatinine and urea were quantified in plasma and urine with QuantiChrom Creatinine and Urea Assay Kits (BioAssay Systems, Hayward, CA).

Blood urea nitrogen (BUN) levels and creatinine clearances (C_{Cr}) were calculated using standard equations, which are: BUN = plasma urea÷2.14. Creatinine clearance (C_{Cr}) was calculated using the following standard equation: $C_{Cr} = (U_{Cr} \times V) \div (P_{Cr} \times t_{min})$; where U_{Cr} = urine creatinine, V = urine volume, P_{Cr} = plasma creatinine, and t_{min} = time of urine collection in minutes. Time of urine collection was 24 hours for each animal.

Isolation of Mitochondria and Measurement of Mitochondrial Permeability Transition

Kidney mitochondria were isolated by differential centrifugation of renal cortical homogenates and mitochondrial swelling was measured as described previously [104]. Briefly, mitochondrial isolation buffer contained (in mmol/L): 140 D-mannitol, 75 sucrose, 1 EGTA, 10 MOPS; and 0.4% BSA, pH 7.4, and a mixture of protease inhibitors (Complete; Roche Diagnostics, Mannheim, Germany). Mitochondrial protein concentration was determined using a DC Protein Assay Kit (Bio-Rad, Hercules, CA). Mitochondrial purity was determined by western blot with an anti-voltage dependent anion channel (VDAC; a mitochondrial marker) antibody (Santa Cruz Biotechnology), and by transmission electron microscopy (Figures 2.1A and 2.1B).

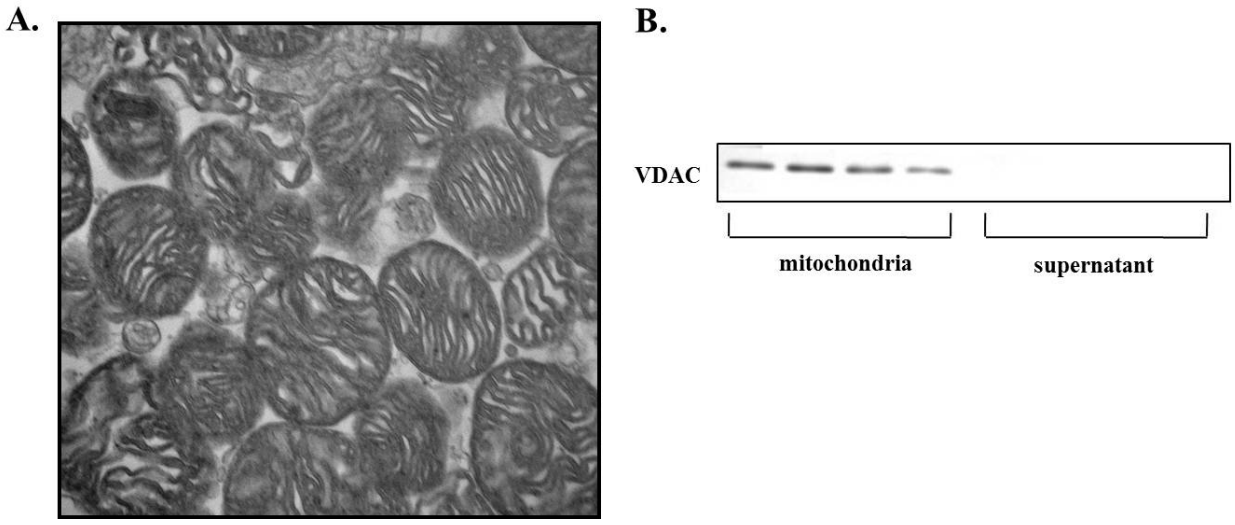

Figure 2.1. Mitochondrial purity as determined by (A) transmission electron microscopy and (B) western blotting. Voltage-dependent anion channel (VDAC) was used as a mitochondrial marker.

Measurement of NAD(P)H-Dependent Superoxide Anion Production

Lucigenin-enhanced chemiluminescence was used to measure NADPH oxidase activity in kidney tissues according to the method of Li *et al* [119].

Determination of Catalase Activity

Catalase activity was measured by the method of Beers and Sizer, as previously described [104, 120].

Determination of Glutathione Peroxidase Concentrations

Glutathione peroxidase concentrations were determined in cortex homogenates and in isolated mitochondria by use of a commercially available kit, according to the manufacturer's protocol (Cayman Chemicals, Ann Arbor, MI).

Measurement of ATP Production

Rates of ATP production were quantified in cortical mitochondria using a commercially available kit (BioVision, Mountain View, CA).

Western Blotting and EMSA

Protein expression in kidney cortical tissue was analyzed by western blot as previously described [116], using anti-p65, anti-p50, anti-IκBα, anti-pIκBα, anti-TNF-α, anti-IL-6, and anti-IL-1β antibodies (Santa Cruz Biotechnology, Santa Cruz, CA Densitometry analyses were performed with Image J software. All membranes were normalized to GAPDH. Data in all western blot figures are expressed as the ratio of the protein density to that of GAPDH. NF-κB p65 DNA binding activity was assessed by electrophoretic mobility shift assay (EMSA), as previously described [121].

RNA Isolation and Real-Time RT-PCR

Total RNA extraction, cDNA synthesis, and real-time RT-PCR were performed as previously described [116].

Immunofluorescence

Immunofluorescence detection of desmin, TNF-α, NOX2, and NOX4 was performed as previously described [122], with minor modifications. For the detection of desmin, NOX2, NOX4, and TNF, slides were incubated overnight at 4°C with a 1:100 dilution of goat polyclonal anti-desmin (Dako North America, Carpenteria, CA), or rabbit polyclonal anti-NOX2, anti-NOX 4, or goat polyclonal anti-TNF (Santa Cruz Biotechnology). Lack of nonspecific staining was confirmed using no primary antibody controls. Analyses of fluorescence intensities were performed using NIH Image J software. In the Image J program, outer borders of glomeruli were traced and immunofluorescence was measured only inside the borders drawn. This method allowed for quantification of glomerular immunofluorescence only, and allowed us to exclude tubules from our analyses.

Electron Paramagnetic Resonance (EPR) Studies

All EPR measurements were performed with a BenchTop EPR spectrophotometer e-scan R (Noxygen Science Transfer & Diagnostics GmbH, Elzach, Germany). Three different spin probes were used for EPR studies. CMH was used to measure total tissue reactive oxygen species (ROS) and superoxide ($O_2^{\bullet-}$), and mitochondrial total ROS and $O_2^{\bullet-}$; CPH was used for measurement of tissue peroxynitrite ($OONO^-$); and PPH was used for mitochondrial hydrogen peroxide (H_2O_2) studies. In this EPR protocol, 'total ROS' represents all reactive oxygen species; however, the major sources trapped by the spin trap used are superoxide, hydrogen peroxide, and hydroxyl radical, with other species as minimal contributors. Small portions (15-20 mg each) of kidney tissue from each animal were minced and placed into four wells of a 24-well tissue culture plate containing 20μM KHB with DF and DETC. Tissue pieces were then washed twice with the same buffer to remove any trace contamination, and incubated at 37^oC with specific spin probes for 30 minutes. The incubation of tissue was terminated by placing the plate on ice. All tissue EPR experiments were conducted at 20°C in disposable capillary tubes as previously described [123], under the following settings: center field g = 2.002; field sweep 9.000G; microwave power 20 mW; modulation amplitude 1.90 G; conversion time 10.24 ms; time constant 81.92 ms; receiver gain 3.17 $^{e+0003}$. Mitochondrial total ROS production experiments were performed at 37°C under 20 mmHg of oxygen partial pressure. The setup of the oxygen concentration in KHB was performed using Gas-Controller NOX-E.4-GC (Noxygen Science Transfer & Diagnostics GmbH, Elzach, Germany). For mitochondrial $O_2^{\bullet-}$ production, the values obtained from incubation with SOD and CMH were subtracted from the values obtained from incubation with CMH only.

Separate spin trap solutions were prepared by dissolving 2.2 mg of CMH, 2 mg of CPH, or 2 mg of PPH in 1 ml KHB (20 mM, pH 7.4), prepared with 25 μM DF and 5 μM DETC. All spin probes are non-toxic. CMH is a cyclic hydroxylamine which is a highly cell permeable spin probe for the quantification of slow-released intracellular and mitochondrial ROS. CPH is an effective, partially cell permeable spin probe for the *in vitro* detection of $ONOO^-$ and other radicals. The spin adduct is resistant to reduction by vitamin C and thiols [123, 124]. PPH is a non-cell permeable spin probe for the detection of mitochondrial H_2O_2 production [125]. ROS released by tissues and mitochondria react with these probes to form stable adducts which can be measured using EPR spectroscopy.

Cortical tissues were incubated at 37°C with 6.6 μl of CMH (200 μM) for 30 minutes for ROS measurement; CMH for 30 minutes, then 1.5 μl of PEG-SOD (50 U/μl) for an additional 30 minutes for $O_2^{\bullet-}$ measurement; or 30 μl of CPH (500 μM) for 30 minutes for $OONO^-$ measurement. Aliquots of incubated probe media were then taken in 50 μl disposable glass capillary tubes (Noxygen Science Transfer and Diagnostics, Elzach, Germany) for determination of total cortical ROS, $O_2^{\bullet-}$, or $OONO^-$ production. Renal cortical mitochondria (approximately 4-6 μg protein) from each animal were probed with CMH for mitochondrial total ROS and $O_2^{\bullet-}$ measurements and with PPH (500 μM) and catalase (50 U/ml) for H_2O_2 measurement. Superoxide detection in mitochondria was confirmed by inhibition of the $O_2^{\bullet-}$ signal with 50 U/ml superoxide dismutase (SOD). After adequate mixing, 50 μl mitochondria were taken in disposable glass capillary tubes. Mitochondrial total ROS, $O_2^{\bullet-}$, or H_2O_2 production was detected using EPR under the settings mentioned above.

Statistical Analyses

Data were analyzed with GraphPad Prism 5 software. All data are presented as means ± SEMs. One-way repeated measures ANOVA with a post-hoc Bonferroni procedure was used to compare differences in blood pressure measurements; one-way ANOVA with a Bonferroni procedure was used to assess renal parameters, EPR measurements, antioxidant activities, and ATP production. Two-way ANOVA with a Bonferroni procedure was used in comparisons of NAD(P)H oxidase-dependent superoxide production rates and mitochondrial swelling assays. Results were considered significant when $p<0.05$.

Results

There were no significant differences in body weights, food intake, or water intake among groups in this study. The average daily delivered dose of PDTC was ~85-105 mg/kg/day in both groups. WKY rats had significantly lower MAP and SBP than SHR at baseline and study end (Table 2.1). SHR+ PDTC animals had lower SBP than SHR by week 6, and these values remained significantly lower than the values in SHR for the remainder of the study (Figure 2.2).

Table 2.1. Mean final body weights and mean baseline and ending arterial and systolic pressures (MAP and SBP, respectively) of study groups.

Group (n=6 each)	Final Body Weight (g)	MAP-Baseline	MAP-Week 15	SBP-Baseline	SBP-Week 15
WKY	384.7±6.08	99.5±1.96*†	94.5±2.84*	121.8±4.62*†	120.2±10.14*
WKY+PDTC	392.3±5.99	99.0±3.40*†	99.9±0.83*	112.8±3.42*†	119.5±6.65*
SHR	375.3±5.95	141.2±6.16	168.9±7.94†	160.4±8.23	196.4±9.76†
SHR+PDTC	387.5±9.26	132.7±10.12*	105.0±1.13*	161.4±8.30	151.4±2.12*

Values are reported as means ± SEM. * $p<0.05$ vs. SHR; † $p<0.05$ vs. SHR+PDTC.

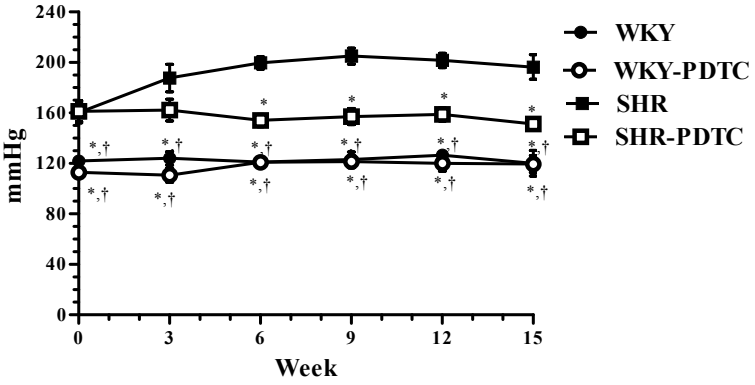

Figure 2.2. Systolic blood pressure trends for each study group. Values are reported as means ± SEM. * $p<0.05$ vs. SHR; † $p<0.05$ vs. SHR+PDTC.

Significant decreases in glomerular filtration rate (GFR) and renal blood flow (RBF) were noted in SHR when compared to other groups. Creatinine clearances (C_{Cr}), which were calculated to verify inulin clearance data, followed the same trend. Additionally, urine albumin, plasma creatinine, and BUN were all significantly higher in SHR than in other groups. Mean values for each parameter appear in Table 2.2.

Table 2.2. Mean values of selected urine and plasma parameters for each study group.

Groups (n=6)	**GFR** (ml/min/g KW)	**RBF** (ml/min/g KW)	**Plasma Cr** (mmol/L)	**BUN** (mmol/L)	**UA** mmol/L/24 hr)	**Alb/Cr**
WKY	0.972±0.104*	9.70±0.791*	0.72±0.04*	16.74±1.13*	441.2±35.7*	0.320±0.022*
WKY+PDTC	1.020±0.049*	11.03±0.610*	0.49±0.13*	11.41±2.21*	345.2±25.7*	0.341±0.006*
SHR	0.641±0.043†	3.83±0.589†	1.71±0.24†	25.18±1.15†	925.6±33.3†	0.511±0.033†
SHR+PDTC	0.938±0.063*	7.70±0.804*	0.51±0.09*	16.78±0.86*	358.7±31.2*	0.334±0.029*

Values represented are means±SEM. *$p<0.05$ vs.SHR; †$p<0.05$ vs.SHR+PDTC.

Total ROS, $O_2^{\bullet-}$, and $OONO^-$ tissue production rates and total ROS, $O_2^{\bullet-}$, and H_2O_2 production rates in mitochondria, as determined by EPR, were all significantly higher in cortical

tissues of untreated SHR than in WKY, WKY+PDTC, and SHR+PDTC animals (Table 2.3). These results indicate that production rates of reactive oxygen and nitrogen species are higher in both the kidney cortex tissue and the cortical mitochondria of the SHR than in the normotensive WKY, and that PDTC attenuates these increases in production. Further, peak values of NAD(P)H-dependent $O_2^{\bullet-}$ production, as measured by lucigenin assay, were significantly higher in the SHR group than in other groups (Figure 2.2).

Table 2.3. Mean production values for tissue total reactive oxygen species (ROS), superoxide ($O_2^{\bullet-}$), and peroxynitrite ($OONO^-$) ; and for mitochondrial total ROS, $O_2^{\bullet-}$, and hydrogen peroxide (H_2O_2), as measured by EPR.

	TISSUE (µmol/mg protein/minute)			MITOCHONDRIA (nmol/mg protein/minute)		
Groups (n=6)	**Total ROS**	**$O_2^{\bullet-}$**	**$OONO^-$**	**Total ROS**	**$O_2^{\bullet-}$**	**H_2O_2**
WKY	0.2132±0.0375*	0.0103±0.0019*	0.00524±0.0101*	0.4386±0.0305*	0.0411±0.014*	0.0032±0.0001*
WKY+PDTC	0.1314±0.0329*	0.0206±0.0041*	0.00510±0.0157*	0.4981±0.0699*	0.0337±0.0124*	0.0031±0.0002*
SHR	0.4615±0.0779†	0.0925±0.0188†	0.01974±0.0392†	1.100±0.1246†	0.2505±0.0649†	0.0063±0.0005†
SHR+PDTC	0.2013±0.0318*	0.0193±0.0043*	0.00641±0.0444*	0.6385±0.0789*	0.0605±0.0166*	0.0041±0.0006*

Values represented are means±SEM. *p<0.05 vs.SHR; †p<0.05 vs.SHR+PDTC.

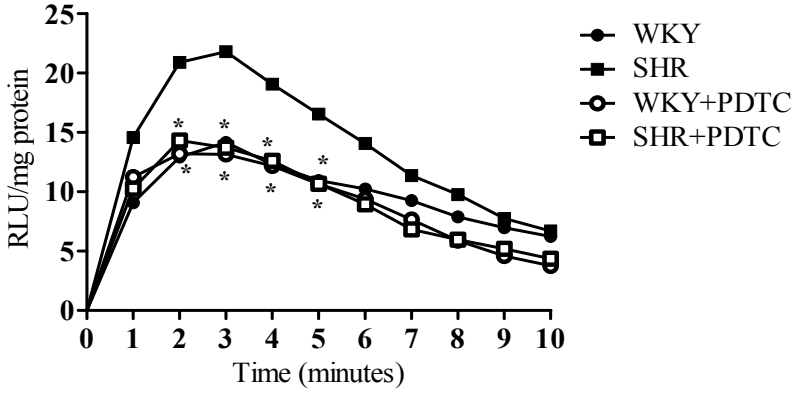

Figure 2.3. NAD(P)H–dependent superoxide production in cortical tissues. * p<0.05 vs. SHR, † p<0.05 vs. SHR+PDTC.

Protein expression levels of desmin (Figure 2.4A), NOX4 (Figure 2.4B), and NOX2 (gp91^{phox}; Figure 2.4C) were all significantly lower in WKY, WKY+PDTC, and SHR+PDTC rats than in SHR; mRNA expression followed a similar trend (Table 2.4). Expression levels of NF-κB p65, p50 and IκBα followed similar trends (Figure 2.5A). SHR also exhibited significantly higher DNA binding activity of NF-κB p65 (Figure 2.5B).

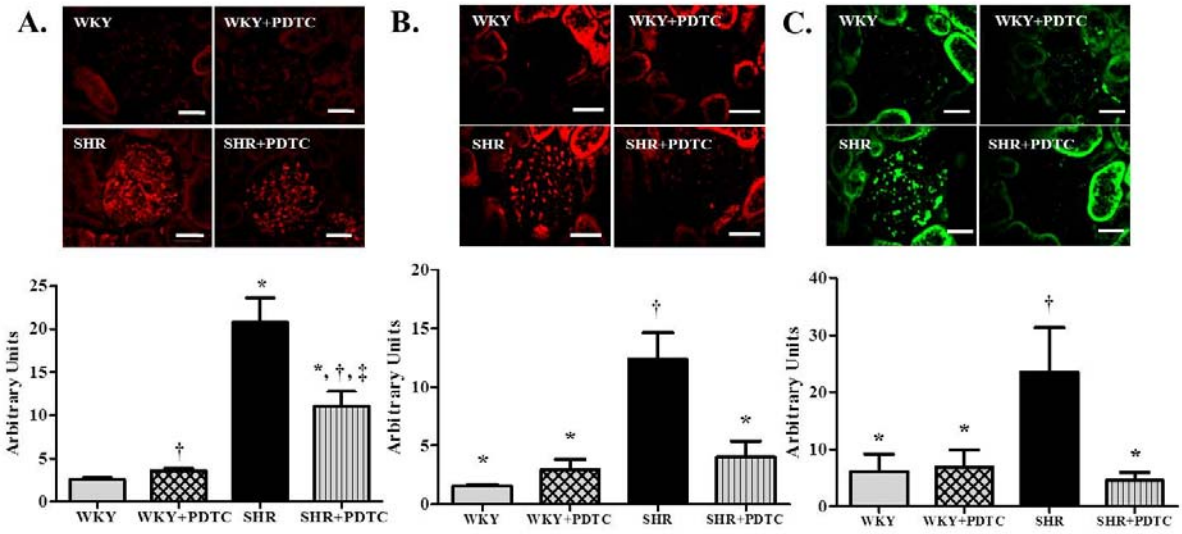

Figure 2.4. Immunofluorescence staining and luminometric analysis for glomerular desmin (A), NAD(P)H oxidase (NOX)2 (gp91phox; B), and NOX4 (C).

Table 2.4. Gene expression values of desmin, NOX homologs, cytokines, IκBα, and NF-κB p65 in cortical tissues of WKY and SHR animals after 15 weeks of PDTC treatment.

Gene	WKY+PDTC	SHR	SHR+PDTC
Desmin	0.839±0.258*	1.737±0.111	0.333±0.223*
NOX2	0.716±0.024*	3.428±0.693	0.251±0.666*
NOX4	0.814±0.356*	2.970±0.291	0.494±0.151*
TNFα	0.695±0.050*	2.901±0.985	1.038±0.147*
IL-1β	0.893±0.025	1.091±0.125	0.873±0.416
IL-6	0.403±0.014*†	1.834±0.183	0.638±0.030*
IκBα	1.261±0.080*	0.372±0.080	1.381±0.197*
NF-κB p65	0.785±0.148*†	1.451±0.084	0.112±0.022*

Values are expressed as mean ± SEM fold change ($2^{-\Delta\Delta Ct}$) vs. WKY control. *p<0.05 vs. SHR; †p<0.05 vs. SHR+PDTC.

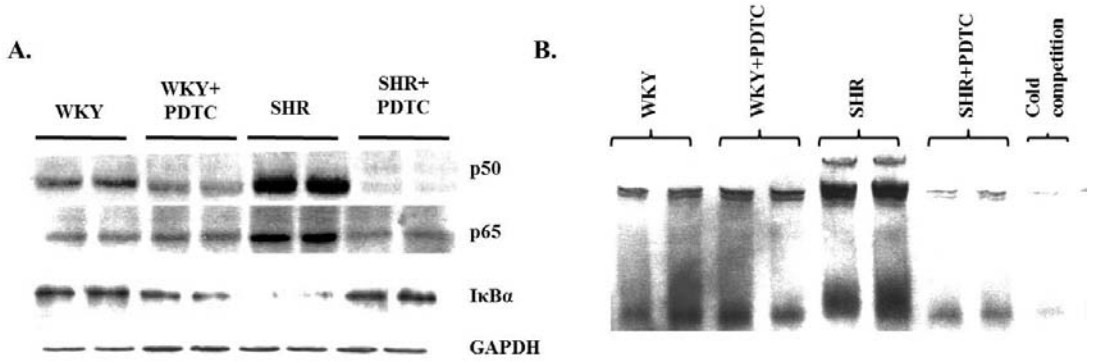

Figure 2.5. Representative western blots for NF-κB p65, p50, IκBα, and GAPDH (housekeeping) in cortical tissues (A) and EMSA for NF-κB p65 DNA binding activity (B). **P* < 0.05 vs. SHR; †*P* < 0.05 vs. SHR+PDTC.

SHR exhibited significantly lower activities of tissue antioxidant levels of catalase and glutathione peroxidase and lower mitochondrial glutathione peroxidase activity (Figure 2.6A), along with significantly decreased ATP production (indicative of electron transport chain damage) (Figure 2.6B). Plasma levels of IL-6 and TNF-α, but not IL-1β, were significantly higher in SHR than in other animals (Figure 2.7A); protein and mRNA expressions of these cytokines followed a similar trend (Table 2.4; Figures 2.7B-2.7D). Increased mitochondrial swelling (indicative of mitochondrial membrane damage) was also observed in SHR (Figure 2.8).

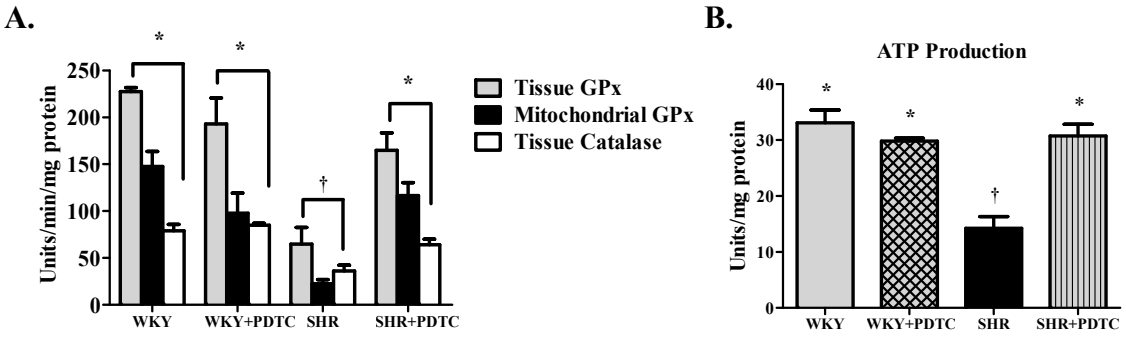

Figure 2.6. Mean enzyme activities for tissue and mitochondrial glutathione peroxidase (GPx) and tissue catalase (A) and mean ATP production rates in experimental groups (B). * p<0.05 vs. SHR, † p<0.05 vs. SHR+PDTC.

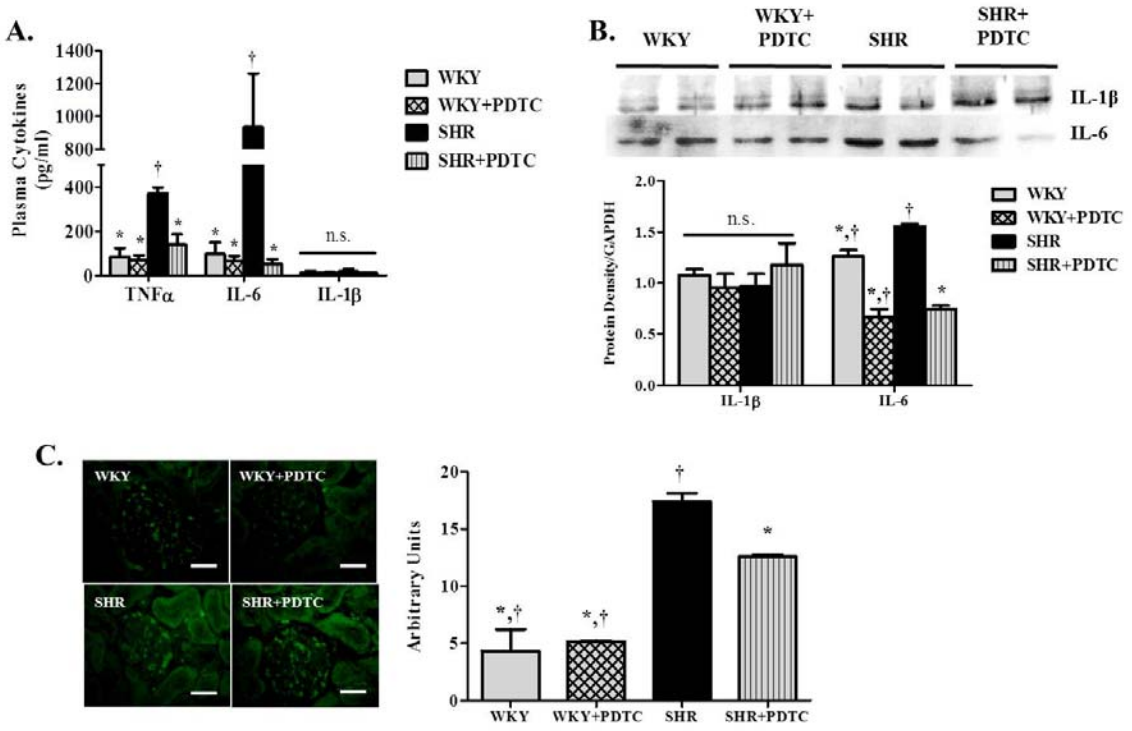

Figure 2.7. Mean plasma cytokine levels (A) and cytokine protein expression levels (B, C) for all study groups. Representative western blots and densitometric analyses for IL-6 and IL-1β appear in (B). Immunofluorescence staining and luminometric analysis for glomerular TNF-α appear in (C). Scale bars = 50 μm. **P* < 0.05 vs. SHR. †*P* < 0.05 vs. SHR+PDTC.

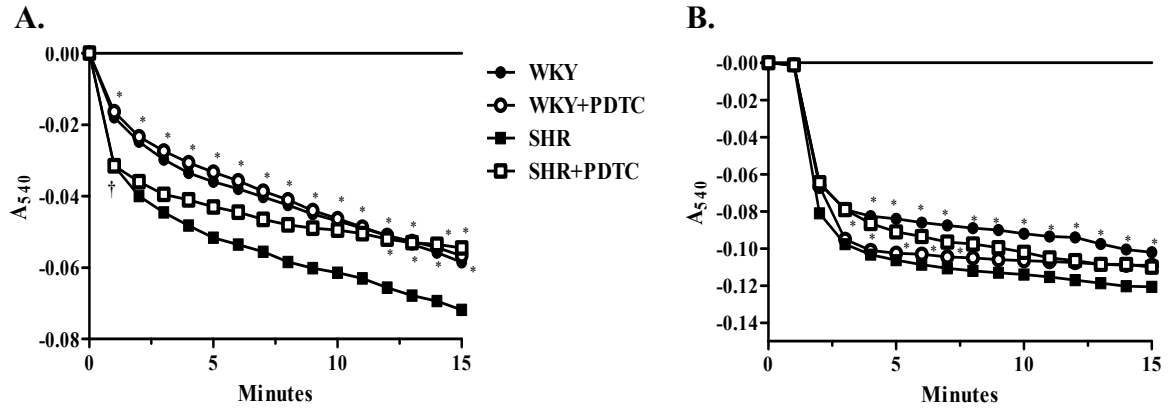

Figure 2.8. (A) Mean basal optical density (OD) and (B) mean OD with 50mM calcium added for mitochondrial swelling assay. * p<0.05 vs. SHR, † p<0.05 vs. SHR+PDTC.

Discussion

In this study, we examined the effects of chronic NF-κB blockade with PDTC on cortical tissue and mitochondrial ROS production in the hypertensive kidney. The salient findings of the present study are: 1) cytosolic and mitochondrial oxidative stress, caused by up-regulation of NF-κB and NF-κB-induced PIC, contribute to renal damage and hypertension in SHR; and 2) NF-κB blockade partially attenuates blood pressure, and normalizes renal function parameters and cytosolic and mitochondrial redox status in SHR. These data suggest that NF-κB plays a role in hypertensive injury in renal cortical tissue and mitochondria by increasing production of PIC and ROS, and that long-term NF-κB blockade can ameliorate these detrimental effects.

We found significant decreases in MAP and SBP in SHR+PDTC rats when compared with untreated SHR, and saw no change in MAP or SBP in WKY treated with PDTC. PDTC treatment also improved GFR, RBF, plasma creatinine levels and BUN, and urinary albumin levels in SHR. Other researchers have used PDTC in hypertensive rat models, and have found similar improvements in blood pressure and end organ damage [112, 126, 127].

Renal and vascular oxidative stress are known to accompany hypertension in the SHR [128]. Increased ROS production, the exact cause of which remains unknown, is thought to be both a cause and a consequence of hypertension [111]. A number of mediators of this oxidative stress have been identified, including PIC (such as TNF-α and IL-6) and angiotensin II (ANGII); both can degrade IκBα to cause NF-κB activation and further increase ROS production. PDTC is believed to exert its inhibitory effects on NF-κB by directly impeding IκBα degradation[129].

PDTC treatment in SHR attenuated the up-regulation of protein and mRNA expression of desmin (a marker of glomerular injury). Desmin expression in most rat strains is confined to mesangial cells; podocytes only express desmin following injury [130]. ROS are known to alter

several signaling cascades in podocytes [131]. Further, NF-κB activation has been shown to upregulate ROS-induced inflammation in mouse podocytes [132]. This evidence, along with our current findings, suggests a critical role for NF-κB in glomerular epithelial injury. NAD(P)H oxidase is the predominant source of ROS production in the renal cortex, and the predominant NOX isoform expressed in the kidney cortex is NOX4 [133]. PDTC administration also attenuated protein and mRNA expression of NOX2 ($gp91^{phox}$) and NOX4 in glomeruli. NAD(P)H-dependent $O_2^{\bullet-}$ production was also decreased in the cortical tissues of PDTC-treated SHR. Taken together, these results suggest that NF-κB-mediated activation of NAD(P)H oxidases, and of desmin, contributes at both the transcriptional and translational levels to the renal damage seen in SHR.

Both mitochondria and NAD(P)H oxidases are important sources of ROS in cells. NAD(P)H oxidases are activated and upregulated in SHR prior to the onset of hypertension in this model [103]. Although $O_2^{\bullet-}$ from NAD(P)H oxidases is considered the major player in glomerular injury, the possible contributory role of mitochondrial ROS in perpetuating renal dysfunction in hypertension cannot be ignored. Recently, Doughan *et al* [134] demonstrated that the full enzymatic activity of NAD(P)H oxidase was required for ANGII-induced mitochondrial damage. Also in that study, NAD(P)H oxidase blockade with apocynin was shown to attenuate ANGII-induced mitochondrial damage in endothelial cells [134]. The same effects on mitochondrial dysfunction were also seen in a study by De Cavanaugh *et al*, which demonstrated decreased mitochondrial oxidant production and improved mitochondrial membrane potential in SHR with angiotensin II receptor blockade [135]. These results suggest that NAD(P)H oxidase-dependent $O_2^{\bullet-}$ can act as an upstream signal to cause increased mitochondrial $O_2^{\bullet-}$ production. This mitochondrial $O_2^{\bullet-}$, along with H_2O_2 which can diffuse out of the mitochondrion, can then

act to further stimulate NAD(P)H activation in a feed-forward mechanism. Further, $O_2^{\bullet-}$ produced from NAD(P)H oxidases can activate NF-κB either directly, or indirectly through an increase in mitochondrial ROS production; however, further studies are needed to elucidate the degree of involvement of both mitochondria and NAD(P)H oxidases in the activation of this ROS-dependent transcription factor.

Until now, no studies have examined the effect of NF-κB blockade on mitochondrial functionality in the hypertensive kidney. We employed EPR, a reliable and sensitive method of measuring and quantifying ROS production, to analyze production of various ROS in both tissue and isolated mitochondria of experimental animals. As expected, PDTC treatment decreased production of total ROS, $O_2^{\bullet-}$, and $OONO^-$ as determined by EPR in the renal cortical tissue of SHR, thereby signifying the role of NF-κB in tissue ROS production.

Mitochondrial total ROS, $O_2^{\bullet-}$, and H_2O_2 production rates were all significantly lower in PDTC-treated SHR. Mitochondrial membrane integrity and ATP production rates were also significantly improved in PDTC-treated SHR. Hypertension is associated with mitochondrial dysfunction in several tissues including the heart and kidney [97, 135]. ROS generated by the mitochondrial electron transport chain (ETC) may act as second messengers to the activation of NF-κB by cytokines such as TNF-α [136]. Also, cells lacking functional mitochondrial ETC show significant down-regulation of NF-κB activation [137], thus reinforcing a role for mitochondrial ROS in activation of NF-κB. However, some caution must be used in interpreting these results, as isolated mitochondria were used. Further studies should employ *in vivo* mitochondrial blockade and ROS measurement; results from these procedures would allow us to provide more representative data in regard to the function of mitochondrial ROS in the hypertensive kidney as a whole.

NAD(P)H-dependent $O_2^{\bullet-}$ and other cytoplasmic ROS can also activate NF-κB, causing a further increase in ROS production, which leads to increased mitochondrial ROS production and further perpetuates this vicious positive feedback cycle. In this study, increased activity and expression of NF-κB and the cytokines TNF-α and IL-6 were associated with increased tissue ROS production (especially that of $O_2^{\bullet-}$), as determined by EPR and lucigenin assay. Increased NF-κB and PIC expression were also associated with increased mitochondrial ROS production and decreased ATP production, both of which suggest a contributory role for mitochondrial dysfunction in hypertensive renal injury. Decreased ATP production is an indicator of ETC dysfunction, as is increased mitochondrial ROS production; damage to the ETC results in free radical leakage, thereby perpetuating mitochondrial damage and ROS production. Chronic NF-κB blockade with PDTC may have attenuated increases in ROS by partially inhibiting the positive feedback between mitochondrial and cytosolic ROS, NF-κB, and NF-κB-regulated PIC in the hypertensive kidney.

Superoxide generated by the mitochondrial ETC can be converted to H_2O_2 in the mitochondrial matrix or in the intermembrane space. H_2O_2 can be detoxified to water by mitochondrial glutathione peroxidase, or to water and oxygen by catalase. These enzymes comprise a complex mitochondrial defense system that is critical in ROS detoxification. In this study, SHR exhibited lower activities of catalase and glutathione peroxidase, and an increased H_2O_2 production rate, as measured by EPR, indicating the impairment of the mitochondrial antioxidant defense system in the presence of increased NF-κB activity. Long-term NF-κB blockade with PDTC restored the activities of these antioxidant enzymes to near control levels in SHR. These results suggest that impairment of mitochondrial antioxidants, combined with

overproduction of mitochondrial ROS, could contribute to the mitochondrial damage seen in hypertensive renal injury.

This study provides a first glance at the role of NF-κB in mitochondrial ROS production in the hypertensive rat kidney. Although this study is the first to quantify mitochondrial ROS in the kidney cortex in the presence or absence of NF-κB blockade, some limitations exist. First, our results suggest an obvious role for mitochondrial ROS in renal injury, but they do not allow us to determine the exact source of mitochondrial ROS overproduction, or the degree of involvement of mitochondrial ROS, since mitochondrial inhibitors were not used. Second, we cannot exclude the possibility that the antioxidant properties of PDTC are also involved in the reduction of ROS production in our study. Lastly, there was a significant decrease in SBP (196.4±9.76 vs. 151.4±2.12) with PDTC treatment; this also could have altered the antioxidant/oxidant parameters measured in this study, thereby contributing to the beneficial effects seen with PDTC. However, the EMSA results and mRNA and protein expression data showing decreased IκBα and increased p65 and p50 in SHR kidney cortex suggest that direct inhibition of NF-κB is responsible, at least in part, for the beneficial effects seen in this study. In an extension of this study that is currently ongoing, we are using mitochondrial inhibitors; this will allow us to better define the role of the mitochondrion in contributing to renal abnormalities in SHR.

In conclusion, the results of the present investigation support a possible role for mitochondrial ROS in hypertensive renal injury (in addition to cytosolic ROS), and suggest that NF-κB-induced PIC negatively affect mitochondrial and tissue ROS production in the hypertensive renal cortex. Successful prevention of renal damage should therefore involve therapies that not only inhibit cytokine-induced NF-κB activation, but also offer mitochondrial

protection from NF-κB-induced PIC and ROS production. Future research should focus on the precise signaling mechanisms by which NF-κB-induced PIC and ROS and mitochondrial ROS interact in the kidney in the setting of essential hypertension.

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CHAPTER 3

CHRONIC EXERCISE PRESERVES RENAL STRUCTURE AND HEMODYNAMICS IN SPONTANEOUSLY HYPERTENSIVE RATS

Introduction

Hypertension-induced renal dysfunction is a significant cause of morbidity and mortality in hypertensive patients, and remains a leading cause of end-stage renal disease in the United States [138]. Current anti-hypertensive treatments are mostly effective in reducing the severity of hypertensive renal disease; however, its progressive clinical course underscores the need for new therapeutic approaches. The benefits of non-pharmacological interventions, such as diet and exercise, on several chronic diseases are well-established. To date, no detailed reports exist that examine the effects of exercise training (ExT) on renal redox status, renal hemodynamics, or renal structure in the hypertensive condition. Therefore, we chose to examine the effects of ExT on renal function and injury and also examined ExT-induced changes in oxidative, nitrosative, and inflammatory parameters in the spontaneously hypertensive rat (SHR), a genetically hypertensive rat model that exhibits many features of human essential hypertension.

We and others have demonstrated that proinflammatory cytokine (PIC) production and several renin-angiotensin system (RAS) components are increased in the hypertensive kidney [139-141]. Reactive oxygen species (ROS) production is also increased in the renal tissue of the SHR. Further, PICs and RAS components have been found to increase ROS production [142-144], which in turn can activate various intracellular signaling pathways, including that of the transcription factor nuclear factor-kappa B (NF-κB) [139]. Activation of NF-κB induces transcription of PIC genes, leading to further increases in ROS production and fostering a cyclic positive feedback mechanism, thereby accelerating the progression of hypertension and its associated renal changes.

Several previous studies have investigated the effects of exercise on hypertension and kidney diseases; however, most of the studies were performed on patients or animals with established disease or the exercise was combined with other interventions [145-149]. However, the effects of ExT on delaying or preventing the progression of hypertension-induced renal injury have not yet been elucidated. The mechanisms by which chronic ExT may affect renal function are unknown, although various mechanisms have been proposed, which include a direct lowering of blood pressure, leading to reduced peripheral vascular resistance [150] and reductions in oxidative stress [149]. Here, we hypothesized that chronic ExT would preserve renal structure and function by modulating oxidative stress and inflammation in the SHR model of hypertension.

Materials and Methods

All procedures in this study were approved by the Louisiana State University Institutional Animal Care and Use Committee and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Animals and Experimental Design

Seven-week-old male normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR), from colonies maintained at the Louisiana State University School of Veterinary Medicine, were used in this study. The original source of the breeder animals was Harlan (Indianapolis, IN). Prior to the initiation of any experimental protocols, five WKY and five SHR were subjected to acute renal clearance experiments as previously published, to obtain baseline values for glomerular filtration rate (GFR) and renal plasma flow (RPF) [139]; animals were euthanized by thiobutabarbital overdose immediately following clearance experiments. Four groups of eight animals each were used for the experimental protocol: sedentary WKY (WKY-S), sedentary SHR (SHR-S), exercise WKY (WKY-E), and exercise SHR (SHR-E).

Animals in exercise groups were subjected to moderate-intensity exercise on a motor-driven treadmill for a period of 16 weeks (5 days per week; 60 min per day at 18 m/min, 0^{o} inclination) which includes an acclimation period of 2 weeks. After acclimation, training intensity was set at approximately 60% of maximal aerobic velocity, which corresponds to moderate intensity exercise (18-20m/min). This protocol is established in our lab and has been used in previous studies .

At the end of the study, six animals from each experimental group were anesthetized and subjected to acute renal clearance experiments [139]. Animals in the exercise groups were sacrificed 24 hours after the last exercise session (at age 24 weeks); sedentary animals were sacrificed at the same age. Animals were euthanized immediately after clearance experiments and kidneys were excised; one kidney from each animal was sectioned, with unstained sections being used for immunofluorescence studies and stained sections being used for histopathological examination. Cortical tissue was separated from the other kidney and stored for later analyses. We performed the following experimental procedures as previously described in Chapter 2: real-time RT-PCR, western blot, electron paramagnetic resonance (EPR) studies, immunofluorescence, antioxidant assays, and statistical analysis. A NF-κB p65 DNA binding assay was also performed, using an assay kit from Active Motif, as previously described [151].

Renal Clearance Experiments

Acute renal clearance experiments were performed according to previously published methods [139].

Glomerular Injury Scoring

For light microscopy, renal tissues were fixed with formalin, embedded in paraffin, cut into 4-5 um sections, and stained with periodic acid-Schiff reagent. All tissue sections were evaluated by a veterinary pathologist that was blinded to experimental conditions. A semi-

quantitative glomerular lesion scoring method was used, which was based upon previously published methods for glomerulosclerosis scoring [152] and expanded to include the following criteria: tubular epithelial metaplasia of Bowman's capsule, glomerulosclerosis, mesangial proliferation, and glomerular capillary basement membrane thickening. Since it is well-accepted that tubular metaplasia is the first evidence of glomerular structural alteration in SHR [153], this was the primary lesion used in our scoring system. One hundred glomeruli in each section were examined and severity of each glomerular lesion was graded from 0 to 4 according to the percentage of glomerular involvement; 0 = no sign of tubular metaplasia, +l = 0 to 25% of glomerulus exhibiting tubular metaplasia, +2 = 25 to 50% of glomerulus exhibiting tubular metaplasia, +3 = 50 to 75% of glomerulus exhibiting tubular metaplasia, and +4 = 75 to 100% of glomerulus exhibiting tubular metaplasia. For each specimen, the grade of the lesion severity determined in this manner was totaled, giving rise to a glomerulosclerosis score for each section examined, which ranges 0 to 400.

Measurement of Cortical Nitrate/Nitrite Production

The reaction of nitric oxide (NO) with oxygen can cause the oxidation of NO, leading to nitrate/nitrite production; therefore, levels of nitrate/nitrite are considered an indirect indicator of NO production. Nitrate/nitrite levels were measured in renal cortical tissues of animals from all experimental groups with a commercially available colorimetric assay kit (Cayman Chemical; Ann Arbor, MI), as previously described [151].

Measurement of Cortical NF-κB p65 DNA Binding Activity

Activated NF-κB translocates to the nucleus, where the p65 subunit binds to DNA to promote transcription. The binding activity of free NF-κB p65 in nuclear extracts was assessed with a NF-κB p65 TransAM ELISA kit (Active Motif, Carlsbad, CA) as per manufacturer's instructions, as previously described [154].

Measurement of Cortical Glutathione and Glutathione Peroxidase Levels

Antioxidant status was assessed in renal cortical tissues of animals from all groups by measurement of reduced and oxidized glutathione and glutathione peroxidase, with commercially available colorimetric assay kits (Cayman Chemical, Ann Arbor, MI), as previously described [144].

Analysis of mRNA Expression by Real-Time PCR

Total RNA isolation from renal cortical tissues, cDNA synthesis and real-time RT-PCR were performed as previously described in detail [139, 151].

Analysis of Protein Expression by Western Blotting

Protein expression in renal cortical tissues was determined by western blot analysis as described previously in detail [139, 151].

Electron Paramagnetic Resonance Spectroscopy

Total ROS, superoxide, and peroxynitrite were measured in renal cortical tissues using electron paramagnetic resonance spectroscopy as described previously in detail [139, 144, 155, 156]. In this EPR protocol, 'total ROS' represents all reactive oxygen species; however, the major sources trapped by the spin trap used are superoxide, hydrogen peroxide, and hydroxyl radical, with other species as minimal contributors.

Immunofluorescence

Immunofluorescence detection of 3-nitrotyrosine (Cayman Chemical, Ann Arbor, MI; 1:100 dilution) in paraffin-embedded kidney sections was conducted as previously described [139].

Statistical Analyses

All data are presented as means ± SEM. For baseline analyses between WKY and SHR, Student's t-tests were used. At study completion, Student's t-tests were used to execute planned

comparisons between WKY-S and SHR-S; WKY-E and SHR-E; and SHR-S and SHR-E groups. In all cases, results were considered significant when $p<0.05$.

Results

Body Weights

There were no significant differences in body weights between WKY and SHR groups at baseline (Table 3.1). However, body weights were significantly lower in both WKY and SHR exercise groups at study end when compared to their sedentary counterparts (Table 3.2).

ExT Preserves Renal Hemodynamics in SHR

No differences in systolic blood pressure (SBP) or mean arterial pressure (MAP) were noted between WKY and SHR at baseline (Table 3.1). At study end, the pressures for SHR-E animals were significantly lower than those for SHR-S animals (Table 3.2). There were also no differences observed in GFR, RBF, or renal vascular resistance (RVR) between the WKY and SHR groups at baseline (Table 3.1). Lower GFR and RBF values and higher RVR values were found in SHR-S rats when compared to SHR-E rats at study end (Table 3.2). There were no significant differences in GFR, RBF, or RVR values between WKY-S and WKY-E animals or between SHR-S and SHR-E animals.

Table 3.1. Baseline hemodynamic data for WKY and SHR (obtained at 7 weeks of age).

	WKY (n=5)	**SHR (n=5)**
BW (g)	193.9 ± 1.61	189.4 ± 2.83
SBP (mmHg)	120.6 ± 3.32	124.7 ± 1.99
MAP (mmHg	106.3 ± 2.94	106.8 ± 2.12
GFR (ml/min/g KW)	1.01 ± 0.04	0.94 ± 0.05
RBF (ml/min/g KW)	6.97 ± 0.33	6.25 ± 0.77
RVR (mmHg/ml/min/g KW)	11.41 ± 1.01	14.89 ± 0.51

Data are presented as mean ± SEM.

Table 3.2. Mean body weights and hemodynamic measurements from rats from all groups (obtained at study conclusion).

Parameter	WKY-S (n=6)	WKY-E (n=6)	SHR-S (n=6)	SHR-E (n=6)
BW (g)	372.4 ± 4.61	323.0 ± 7.64$^{\$}$	370.8 ± 2.63†	314.4 ± 11.69*
SBP (mmHg)	121.1 ± 2.37*	130.7 ± 2.36	189.2 ± 3.47†$^{\$}$	144.8 ± 3.50*
MAP (mmHg)	93.9 ± 1.83*	98.6 ± 2.21	163.4 ± 9.30†$^{\$}$	120.0 ± 4.73*
GFR (ml/min/g KW)	0.90 ± 0.09*	0.90 ± 0.05	0.45 ± 0.08†$^{\$}$	0.73 ± 0.05*
RBF (ml/min/g KW)	8.26 ± 1.11*	7.24 ± 0.79	3.58 ± 0.45†$^{\$}$	7.51 ±0.58*
RVR (mmHg/ml/min/g KW)	13.61 ± 1.61*	16.22 ± 0.49	42.49 ± 7.81†$^{\$}$	15.96 ± 2.84*

* $p<0.05$ vs. SHR-S; † $p<0.05$ vs. SHR-E; $^{\$}$ $p<0.05$ vs. WKY-S.

ExT Preserves Glomerular Morphology in the SHR Kidney

PAS-stained kidney sections from rats (n=5 from each group) were examined at the end of the study by a veterinary pathologist who was blinded to the experimental conditions. One hundred glomeruli from each section were scored. Representative photomicrographs of glomeruli for each lesion score appear in Figures 3.1A – 3.1E. There was no significant difference in glomerular lesion scores for WKY or SHR at baseline (WKY score 27 ± 1.44 and SHR score 34 ± 2.61). SHR-S rats had significantly higher glomerular lesion scores than WKY-S and SHR-E rats at the completion of the study (Figure 3.1F). No differences in scores were found between WKY-S and WKY-E rats.

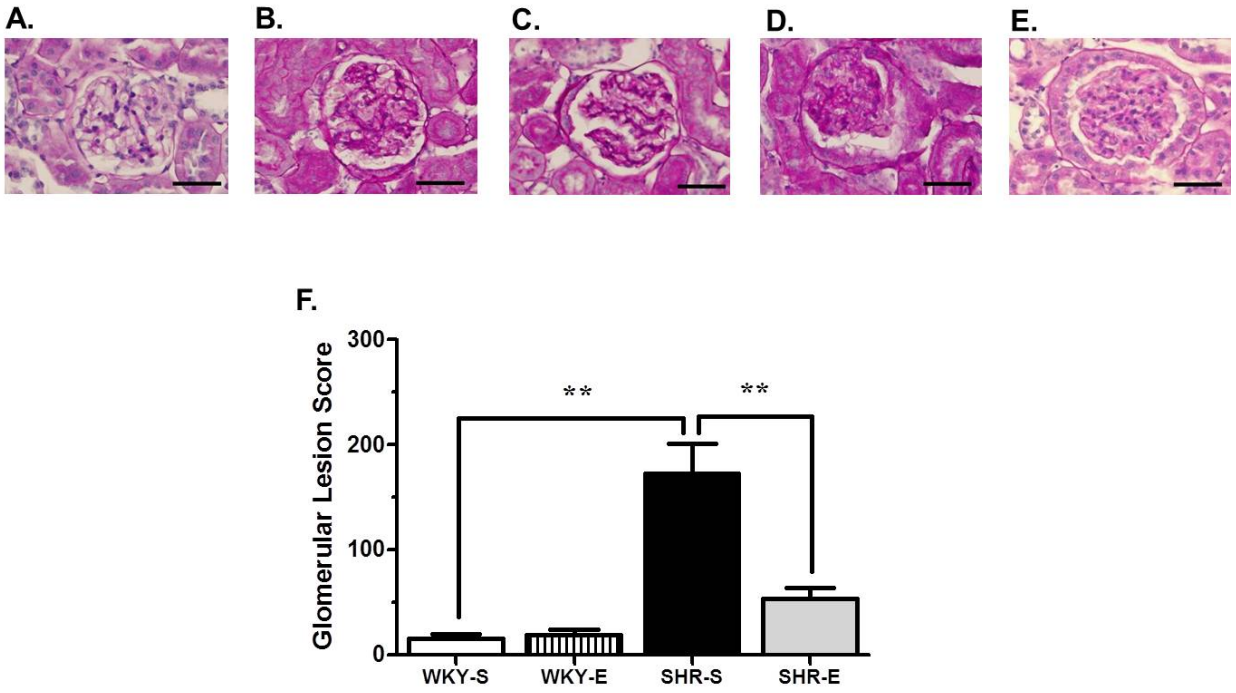

Figure 3.1. Representative photomicrographs for scoring of glomerular injury at study completion. Scale bars = 50 μm. A) Grade 0 – glomerulus with no lesions; B) Grade 1 – 0-25% of glomerular area affected; C) Grade 2 – 25-50% of glomerular area affected; D) Grade 3 – 50-75% of glomerular area affected; E) Grade 4 – 75-100% of glomerular area affected. F) Average glomerular lesion scores for each experimental group (n=5 animals per group). * $p<0.01$; ** $p<0.05$; ***$p<0.001$.

ExT Decreases Total ROS and Superoxide Production in the SHR Kidney

We measured total ROS and superoxide production rates using EPR spectroscopy in cortical tissues from rats (n=6 per group) in all experimental groups. Production rates of both species were significantly decreased in cortical tissues of SHR-E rats when compared to SHR-S rats (Figures 3.2A and 3.2B). Significant differences in total ROS measurements were found between WKY-S and WKY-E rats, but not between WKY-E or SHR-E rats. No significant differences superoxide measurements were found between WKY-S and WKY-E rats or between WKY-E and SHR-E rats.

ExT Decreases Peroxynitrite Production and NT Formation in the SHR Kidney

We measured cortical peroxynitrite levels in all rat groups (n=6 per group) using EPR spectroscopy. We also examined expression of 3-nitrotyrosine (NT), a footprint of peroxynitrite formation, via immunofluoresence. In the SHR-E rats, peroxynitrite production rates were significantly decreased and NT expression levels were lower when compared to those of SHR-S rats (Figures 3.2C and 3.2D, respectively).

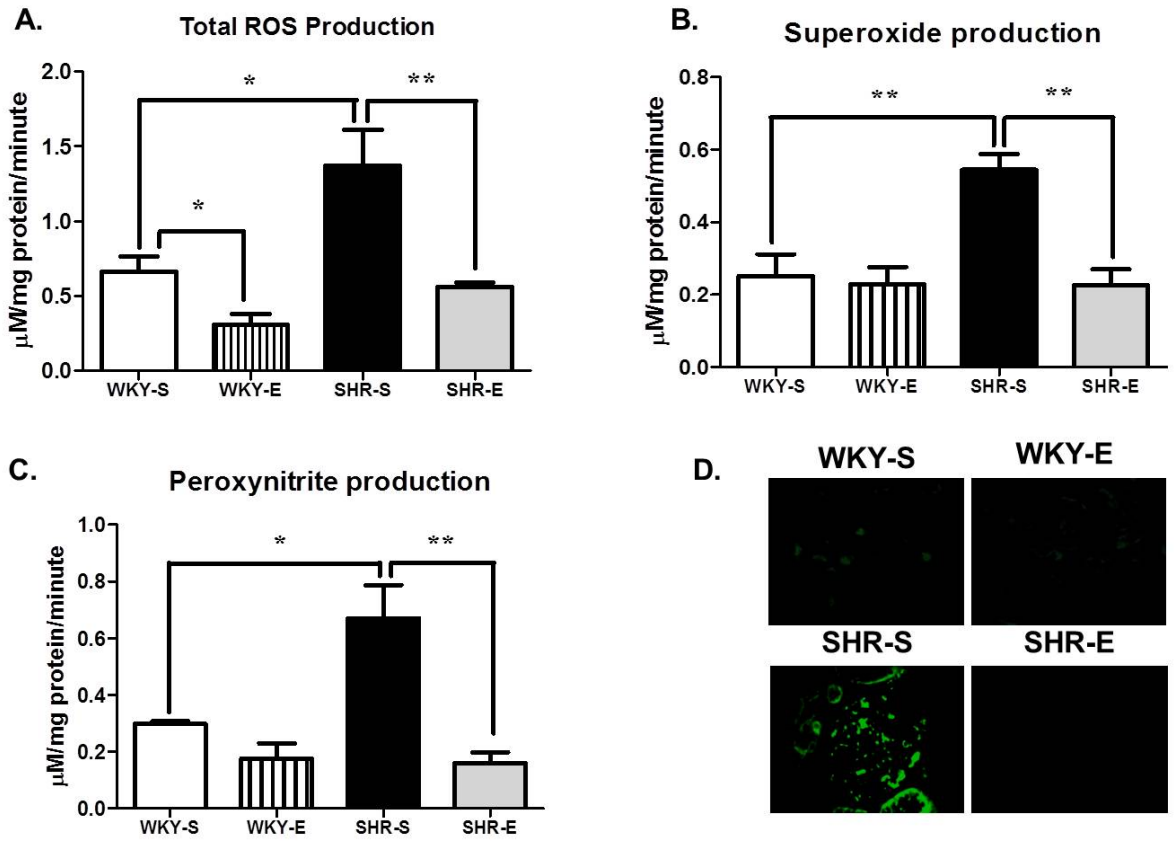

Figure 3.2. Free radical production rates as determined by electron paramagnetic resonance spectroscopy, and immunofluorescence staining for 3-nitrotyrosine. A) Total reactive oxygen species (ROS), B) superoxide, and C) peroxynitrite production rates in renal cortical tissues of rats from each experimental group, D) Immunofluorescence for 3-nitrotyrosine, an indirect indicator of peroxynitrite formation. * $p<0.01$; ** $p<0.05$; ***$p<0.001$.

ExT Improves Antioxidant Status in the SHR Kidney

We measured glutathione peroxidase (GPx) and reduced and oxidized glutathione (GSH and GSSG, respectively) in cortical tissues from each experimental group (n=5 per group), using commercially available kits. Levels of GPx and both GSH and GSSG were significantly lower in SHR-S animals than in any other group (Figures 3.3A-3.3D). Exercise treatment increased GPx, GSH, and GSSG levels in SHR animals.

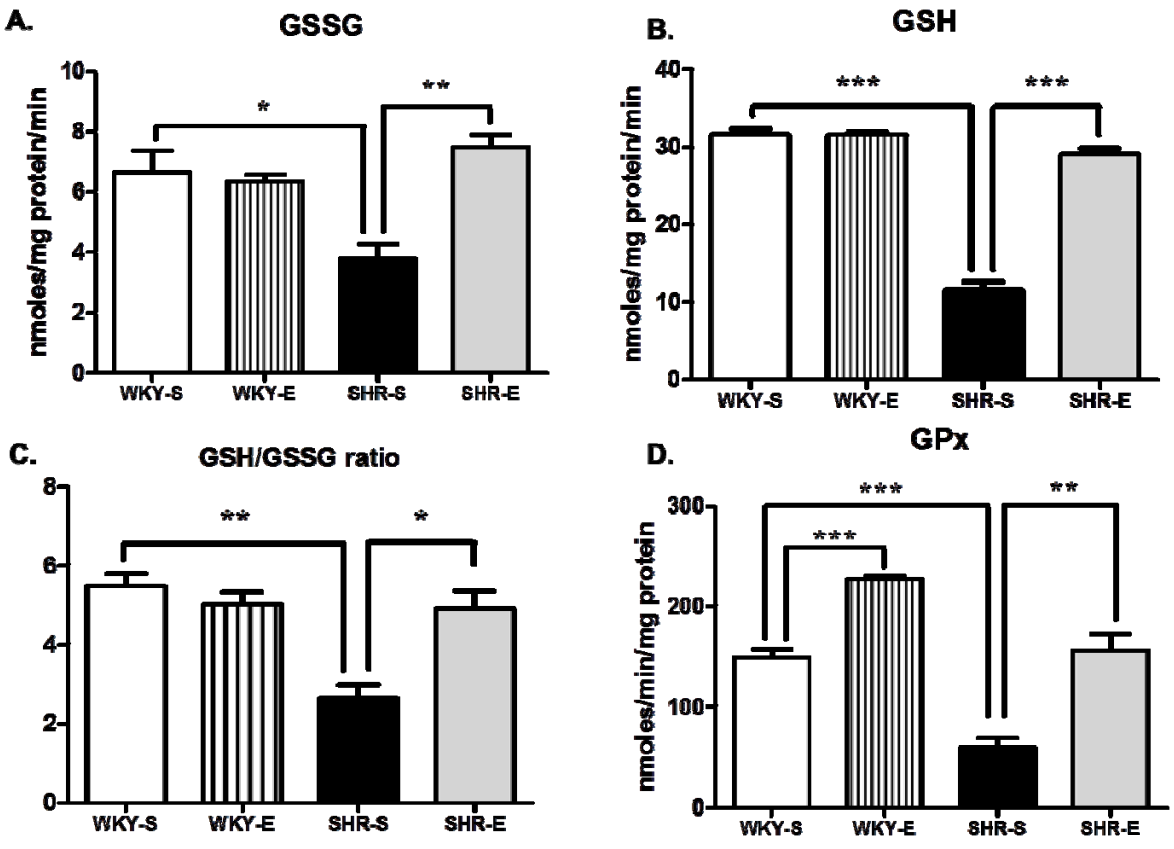

Figure 3.3. Levels of reduced glutathione (GSH; A) and oxidized glutathione (GSSG; B), GSH/GSSG ratio (C), and glutathione peroxidase (GPx; D) as measured in cortical tissues. * $p<0.01$; ** $p<0.05$; ***$p<0.001$.

ExT Decreases NF-κB Activity and TNF-α Expression in the SHR Kidney

Renal cortical NF-κB p65 DNA binding activity was measured in tissues from all groups (n=6 per group). Rats from the SHR-S group had significantly higher cortical NF-κB activity than WKY-S rats (Figure 3.4A). The SHR-E rats had NF-κB activity levels comparable to the WKY-S and WKY-E rats, suggesting that chronic ExT prevents the increase in cortical NF-κB activity seen in SHR. Since the proinflammatory cytokine tumor necrosis factor-alpha (TNF-α) acts through a NF-κB-dependent pathway, we measured protein and gene expression of this cytokine in the cortical tissues of animals from all experimental groups. Expression of TNF-α was significantly increased in SHR-S rats, but was completely normalized in SHR-E rats (Figures 3.4B and 3.4C).

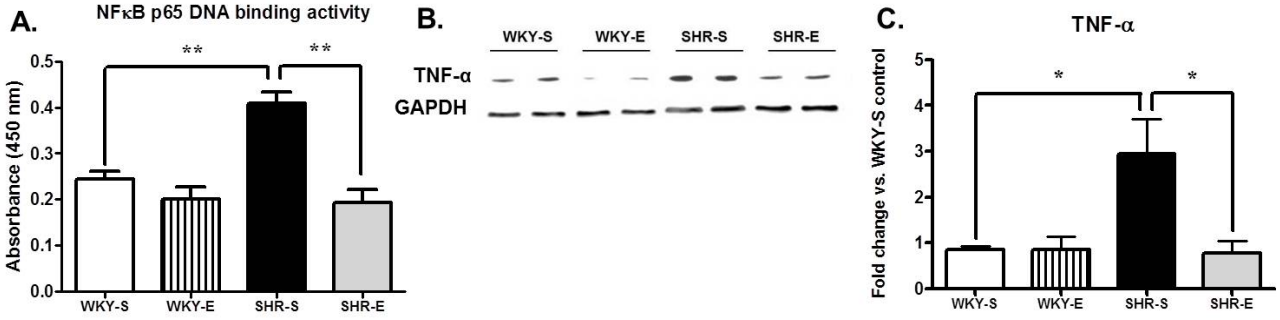

Figure 3.4. NF-κB p65 DNA binding activity (A) and protein (B) and mRNA (C) expression levels of TNF-α in renal cortical tissues. * $p<0.01$; ** $p<0.05$; ***$p<0.001$.

ExT Alters NO Production and NOS Isoform Expression in the SHR Kidney

Protein and gene expression levels of eNOS and iNOS were measured in all experimental groups at study completion (Figures 3.5A and 3.5B, respectively). Cortical nitrate/nitrite levels (indirect indicator of NO production) were also measured (Figure 3.5C). Both protein and gene expression levels of eNOS, along with NO production, were decreased in SHR-S rats, while iNOS levels were significantly increased. These effects were normalized in the SHR-E animals.

ExT Alters Expression of RAS Components in the SHR Kidney

Protein (Figure 3.6A) and gene expression (Figure 3.6B) levels of ACE, ACE2, AT-1 receptor (AT-1R), and Mas receptor (MasR) were measured in all experimental groups at study completion. Circulating AngII levels were also measured in plasma samples from animals from all experimental groups at study end (Figure 3.6C). Both protein and gene expression levels of ACE and AT-1R were higher in SHR-S rats, while ACE2 and MasR levels were significantly lower. Similarly, AngII levels were also elevated in SHR-S animals. In SHR-E animals, ACE2 and MasR expression levels were elevated, while ACE and AT-1R expression levels were decreased. Circulating AngII levels were also lower in SHR-E animals.

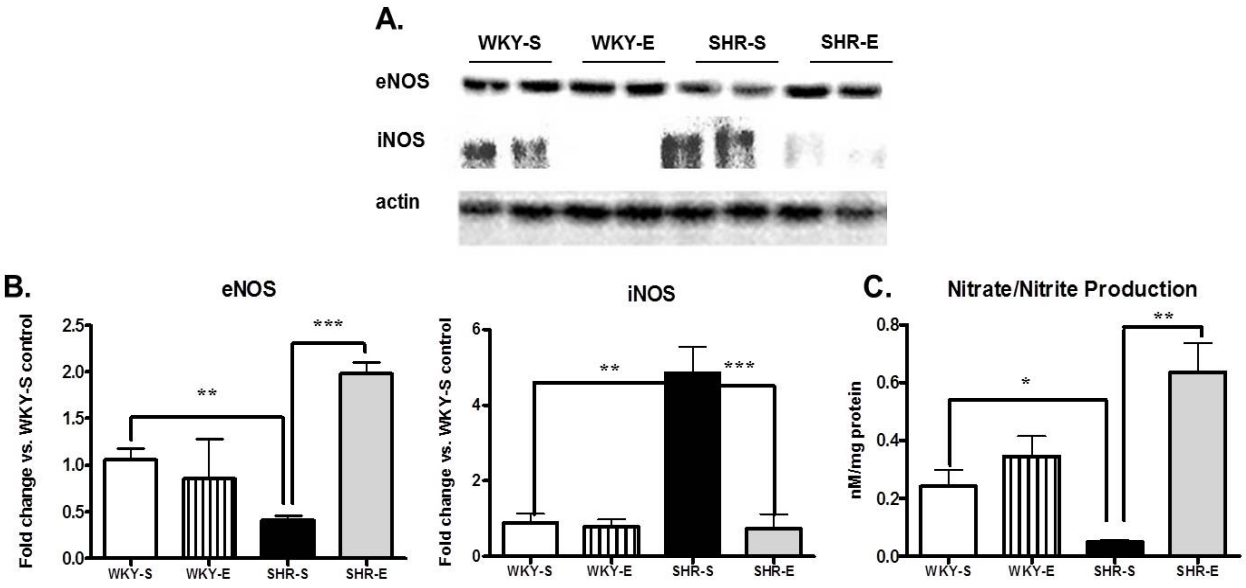

Figure 3.5. Protein (A) and mRNA expression (B) of eNOS and iNOS, and NO production (C) as assessed by nitrate/nitrite measurement, in kidney cortical tissues from each group. *$p<0.01$; ** $p<0.05$; ***$p<0.001$.

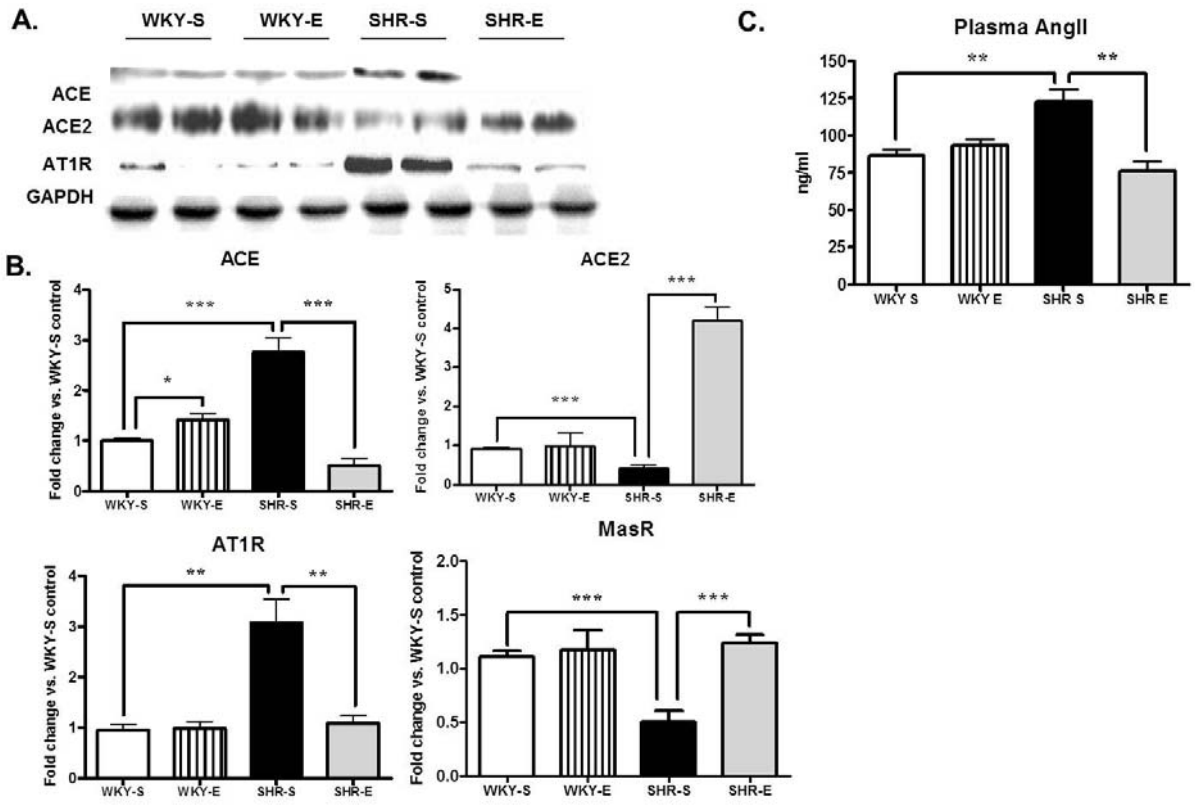

Figure 3.6. A) Protein and B) mRNA expression of various renin-angiotensin system components and C) mean plasma ANGII levels. * $p<0.01$; ** $p<0.05$; ***$p<0.001$.

Discussion

Primary (essential) hypertension remains a major cause of morbidity and mortality in Western society, and is the second leading cause of end-stage renal disease in the United States [157]. The most effective way to avoid the development of hypertension-induced renal injury is to prevent hypertension or to delay its progression. Current pharmacological therapies have proven beneficial in the treatment of hypertension, but the focus has recently shifted to include non-pharmacological approaches, such as exercise, as adjunct therapies to prevent or mitigate hypertension and its end-organ effects. Here, we examined the effects of ExT on renal function and injury and assessed exercise-induced changes in oxidative, nitrosative, and inflammatory parameters in SHR. We initiated chronic ExT at 7 weeks of age, an age when SHR are still

normotensive and have normal renal hemodynamic indices. We also performed baseline measurements of arterial pressures and renal hemodynamic parameters in 7-week old WKY and age-matched SHR, with no statistically significant differences found between strains for any of the parameters measured (Table 1). Results from our endpoint studies demonstrate that chronic, moderate-intensity ExT prevents renal oxidative stress and inflammation, maintains renal antioxidant defense, and modulates both intrarenal and extrarenal renin-angiotensin system components in SHR. Chronic exercise also resulted in a delayed increase in body weight, prevented the development of severe hypertension, and preserved renal structure and renal hemodynamics in these animals.

Oxidative stress is characteristic of the adult SHR, and has been shown to precede the development of hypertension in this rat strain by several weeks [158, 159]. Conversely, hypertension has been shown to cause oxidative stress in the kidney [160]. This self-perpetuating cycle, if left unchecked, can lead to progressive renal disease. Several key mediators of renal oxidative stress have been identified, including PIC and the effector peptide of the renin-angiotensin system, AngII; both can cause activation of the key redox-sensitive transcription factor, NF-κB, and increase production of ROS and reactive nitrogen species (RNS), such as superoxide and peroxynitrite, respectively. These ROS/RNS themselves can increase NF-κB activity, leading to further oxidative/nitrosative insult and RAS activation, which perpetuates this vicious positive feedback cycle and accelerates hypertension-induced renal damage [139]. A schematic representation of this cycle appears in Figure 3.7.

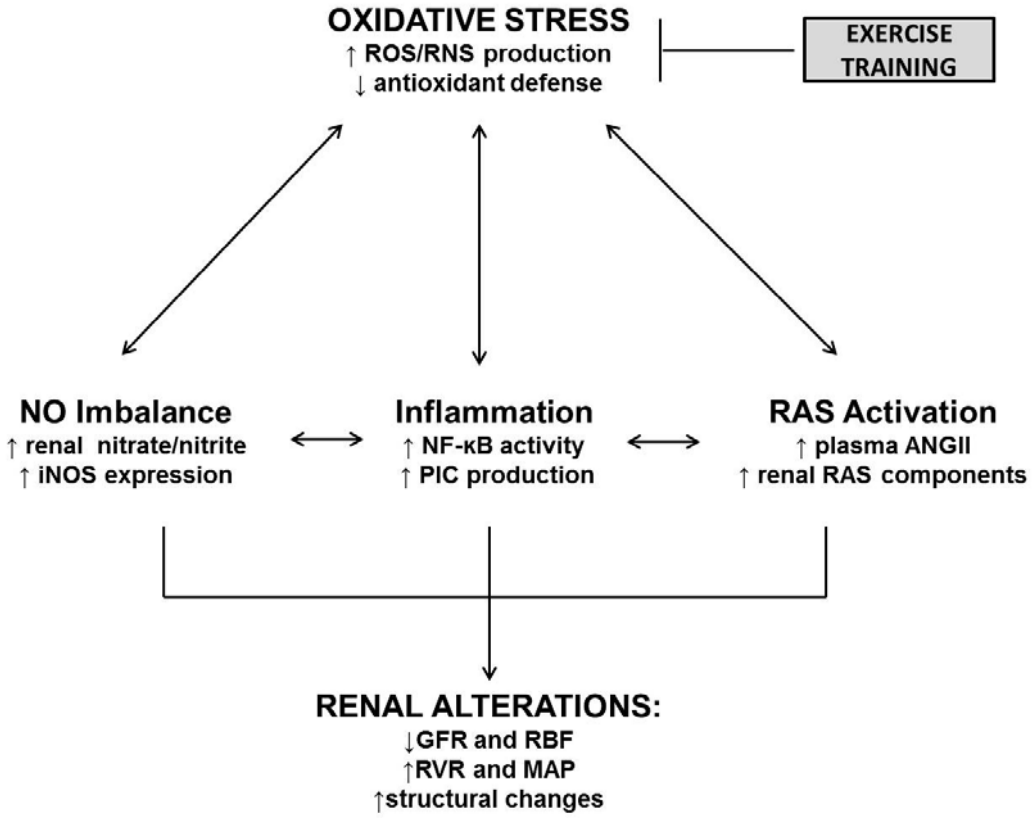

Figure 3.7. Schematic depiction of the interactions of oxidative stress, inflammation, RAS activation, and NO imbalance in the pathogenesis of hypertensive renal injury, and the effects of exercise training on this vicious positive feedback cycle.

NF-κB can induce and respond to oxidative stress; when activated, NF-κB can activate the promoters of two proinflammatory molecules - TNF-α [161] and iNOS [162]. A key finding in the present study is that NF-κB p65 DNA binding activity, along with cortical protein and gene expression levels of iNOS and TNF-α, did not increase in the SHR kidney when ExT was initiated prior to development of hypertension, while all three parameters increased significantly in sedentary SHR. Conversely, gene and protein expression levels of eNOS were significantly higher in SHR-E animals then in SHR-S animals. Further, chronic ExT prevented the increases normally seen in cortical total ROS, superoxide, and peroxynitrite production rates in SHR. Our current finding that decreased oxidative stress is associated with decreased TNF-α expression and decreased NF-κB activity in SHR-E rats raises the possibility that decreased PICs might be responsible for the exercise-induced decrease in oxidative stress in SHR.

Oxidative stress, by definition, involves an excess of free radicals; in the case of renal oxidative stress during hypertension, the predominant free radical is superoxide, which is mostly produced by NADPH oxidases [163]. We have previously demonstrated an up-regulation in NADPH oxidase expression and activity in the cortical tissues of SHR; this was associated with a decline in renal hemodynamic parameters and with increased arterial pressure [139]. In addition to its direct detrimental effects, superoxide can interact with NO to form the highly cytotoxic peroxynitrite radical. Peroxynitrite can then react with tyrosine residues in various proteins to generate NT [164, 165]. Although NT can be formed from reactions that do not include NO [166], the contribution of these reactions to total tissue NT abundance is minimal; thus, tissue NT abundance is largely considered a function of the interaction between superoxide and NO [164]. In this study, the increase in arterial blood pressure and alterations in renal hemodynamics seen in sedentary SHR were accompanied by cortical accumulation of NT and increased tissue production rates of total ROS, superoxide, and peroxynitrite. These findings were also accompanied by a marked reduction in renal cortical NO metabolites, which is suggestive of diminished NO bioavailability, likely resulting from an enhancement of NO inactivation by superoxide. We also found decreased GPx and GSH levels and decreased GSH/GSSG ratio in cortical tissues of SHR-S animals, which is indicative of insufficient antioxidant status. Lee and colleagues recently found similar impairments in the glutathione system in SHR from the age of 8 weeks [167]; our results suggest that this impairment continues through age 24 weeks, and that ExT prevents these alterations in antioxidant status.

In exercised SHR, there was a delayed, moderate increase in arterial pressure and a normalization of cortical NO metabolites. Further, SHR-E exhibited no evidence of cortical NT accumulation and no appreciable increases in tissue production rates of total ROS, superoxide, or peroxynitrite. These results were associated with a preservation of renal hemodynamics and

renal antioxidant status in these animals, and with alterations in cortical gene and protein expression levels of iNOS and eNOS. Our results are in line with a previous report from our lab, where the same ExT regimen resulted in preserved cardiac function, decreased ROS formation, decreased iNOS expression, and increased NO metabolites in the SHR myocardium [151]. Taken together, these observations suggest that chronic ExT can decrease the severity of hypertension and its associated alterations in renal hemodynamics in SHR by ameliorating the renal oxidative stress known to exist in these animals. These beneficial effects seem to involve preservation of redox status and an improvement in NO bioavailability.

Activation of the RAS and the resulting AngII-induced pressor response are key mediators of renal damage [168, 169], and have renal pro-oxidant and proinflammatory effects which negatively alter renal hemodynamic parameters [168, 170]. For example, AngII can antagonize the effects of NO [171], potentiate superoxide production [170], and activate NF-κB [172]; these actions of AngII represent another arm of the vicious positive feedback cycle involved in hypertension-induced renal injury. We measured protein and gene expression levels of several RAS components (AT-1R, ACE, ACE2, and MasR) and circulating AngII levels to assess the possible involvement of the RAS in the renoprotection afforded by ExT in this study. We found down-regulation of AT-1R and ACE and decreased plasma AngII, with concomitant up-regulation of ACE2 and MasR, in exercised SHR when compared to SHR-S animals. These findings were associated with renal hemodynamic improvements, decreased cortical NF-κB activity, and improved cortical redox status in SHR-E animals. Overall, our results indicate a role for decreased RAS activation in the renoprotective effects of ExT in SHR.

The normal parietal tissue of Bowman's capsule consists of simple squamous epithelium [153]. Tubular metaplasia of Bowman's capsule has been well-characterized in SHR and in aging male Sprague-Dawley rats [153, 173, 174]; however, the exact cause of such metaplasia

remains uncertain. Metaplasia usually occurs in response to chronic inflammation and allows for substitution of cells that are better able to survive under circumstances in which a more fragile cell type might succumb. Although the normal squamous epithelium that lines Bowman's capsule is robust and more resistant to many insults than is cuboidal epithelium, local changes in cytokine, growth factor, or extracellular matrix components arising from either increased glomerular pressure or proteinuria may favor metaplasia to renal tubular epithelium [175]. In this study, a higher incidence of tubular metaplasia was seen in SHR-S glomeruli than in glomeruli from any other group, while the incidence of metaplasia in SHR-E glomeruli was similar to that of WKY-S and WKY-E animals. These differences were not present in WKY or SHR that were sacrificed at 7 weeks of age, suggesting that the metaplasia seen in SHR-S glomeruli at study completion may indeed be the result of increased pressure and increased inflammation, as previously suggested [153]. The absence of significant structural alterations in SHR-E kidneys was associated with improved renal hemodynamics and decreased inflammation, while the increased incidence of metaplasia in SHR-S kidneys was associated with a decline in renal hemodynamics and an increase in inflammation. These results suggest that ExT can decrease the incidence of pathological changes in SHR kidneys, possibly through a delay in progression of hypertension and attenuation of inflammation.

We have previously reported an association of PICs and their transcription factor, NF-κB, with oxidative stress and hemodynamic alterations in the adult SHR kidney [139]. Given the results from that report and our current results, it is plausible to suggest that the lower NF-κB activity levels seen in SHR-E animals may be attributable to reductions of PIC and ROS production and of RAS activation, and thus lead to disruption of the positive feedback cycle involved with hypertension-induced renal dysfunction. This study is the first to provide mechanistic evidence surrounding the effects of chronic exercise on NF-κB activity, oxidative

stress, hemodynamics, and structure in the SHR kidney. Our results suggest a major role for exercise training in modulating hypertensive renal injury via decreases in inflammation, oxidative stress, and RAS activation, and suggest that chronic, moderate-intensity ExT may be a cost-effective non-pharmacological approach to preventing hypertension and preserving renal hemodynamics in susceptible patients.

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CHAPTER 4

A BLUEBERRY-ENRICHED DIET ATTENUATES NEPHROPATHY IN A RAT MODEL OF HYPERTENSION VIA REDUCTION IN OXIDATIVE STRESS

Introduction

Oxidative stress produced by overproduction of reactive oxygen species/reactive nitrogen species (ROS/RNS) or inefficient antioxidant defenses appears to be involved in the development and progression of hypertension and hypertension-induced renal injury [176, 177]. The detrimental role of ROS/RNS in hypertension-induced renal injury has fostered an increased interest in the therapeutic potential of antioxidants; however, the majority of studies thus far have employed synthetic antioxidants to prevent or attenuate the detrimental effects of ROS both *in vivo* and *in vitro*. Recently, attention has been directed to natural products as sources of antioxidants [178]. Most plant cells contain antioxidant mechanisms to detoxify free radicals, which are produced during normal cellular metabolic processes; exogenous stimuli can also promote free radical production in plants [179]. In particular, small berry fruits have been demonstrated to have high contents of several antioxidant compounds, including anthocyanins and other phenolics. These metabolites function to protect plants against photodynamic reactions by quenching ROS, and have been suggested to have protective effects against several human diseases [180, 181].

Blueberries (BB) have among the highest antioxidant capacities of fruits and vegetables tested to date, and contain polyphenols such as anthocyanins, anthocyanadins, and isoflavones [182]. BB-enriched diets and BB extracts have been shown to attenuate and even improve age-related behavioral and neuronal deficits in rodents [183-186]. BB-enriched diets can also attenuate proinflammatory cytokine production in rat glial cells [187] and protect the rat heart

from ischemia [188]. Additionally, hypertensive rats on BB-supplemented diets exhibit significantly lower systolic and mean arterial pressures and renal nitrite content [189]. Therefore, it is plausible to suggest that BB supplementation of the diet may have a tissue-protective effect in various pathologic conditions. In this light, the general objective of the current study was to assess the chronic effects of a BB-enriched diet on blood pressure (BP) and renal hemodynamics in a rat model of hypertension-induced renal injury. The hypothesis was that the BB supplementation of the diet would reduce oxidative stress and thus attenuate renal damage. As an extension of this hypothesis, rats were also subjected to a short-term (2-day) exposure of the BB-supplemented diet to determine whether a hormetic effect would be observed. Hormesis has been proposed as the mechanism mediating the protective effects of many plant products [190, 191]. Specifically, the hypothesis was that during short-term exposure, increased ROS/RNS production would be observed which would lead to upregulation of antioxidant defense mechanisms to enhance long-term protection.

Materials and Methods

Experiment 1: Chronic Feeding Studies

Forty-eight male stroke-prone spontaneously hypertensive rats (SHRSP) and thirty-two male normotensive Wistar-Kyoto (WKY) rats were used for chronic feeding studies. Rats were 7 weeks old with baseline body weights between 130 and 150 grams. Rats were randomly divided into four diet groups for each chronic study: WKY cornl (WC), WKY blueberry (WBB), SHRSP corn (SC), or SHRSP BB (SBB). Animals were fed corn or BB-enriched diets for 6 weeks or 12 weeks. All animals were subjected to acute renal clearance experiments, as previously described [176], at the end of the 6-week or 12-week feeding periods. Rats were

euthanized immediately following renal clearance experiments; kidneys were excised, and cortex and medulla separated for analyses.

Experiment 2: Short-Term Feeding Studies

For short-term feeding studies to evaluate hormetic effects, 24 additional 7-week-old male SHRSP were used. Rats (n=12 in each group) were fed corn or BB-enriched diets for 2 days. Rats were euthanized after which heart, brain, kidney, and liver tissues were collected. In both 2-day and chronic studies, fresh tissues were used for electron paramagnetic resonance (EPR) spectroscopy studies, and frozen tissues were used for antioxidant studies

Diets

Diets were prepared by Harlan Teklad (Madison, WI) using a reformulated NIH-31 diet by adding 20 g/kg lyophilized BB or 20 g/kg dried corn. To prepare the 2% BB diet, the berries were homogenized in water, centrifuged, lyophilized and added to the NIH-31 rodent chow. The amount of corn in the corn diet was adjusted to compensate for the added volume of BB [192], in order to make the two diets isocaloric [193]. Food consumption was measured weekly for the chronic feeding studies by weighing feed before placing it in each cage, and subtracting the weight of remaining feed at the end of each week. Rats maintained on BB diets for 6 weeks consumed an average of 371 mg/day (WBB) or 374 mg/day (SBB) of lyophilized blueberries, roughly equivalent to 4.1g/day or 4.2 g/day, respectively, of fresh blueberries. Rats maintained on BB diets for 12 weeks consumed an average of 397 mg/day (WBB) or 399 mg/day (SBB) of lyophilized blueberries, roughly equivalent to 4.4 g/day or 4.5 g/day, respectively, of fresh blueberries.

Blood Pressure Measurements

In rats from all chronic feeding groups, tail blood pressures (BP) were measured noninvasively using a Coda 6 Volume-Pressure Recording System (Kent Scientific, Torrington, CT), as previously described [194]. Briefly, eight unanesthetized rats from each group were warmed to an ambient temperature of 30°C by placing them in a holding device mounted on a thermostatically controlled warming plate. Tail cuffs were placed on animals, and animals were allowed to acclimate to cuffs for 10 minutes prior to each pressure recording session. Each session consisted of 30 cycles. BP was measured on five consecutive days each week, and values were averaged from at least six consecutive cycles. BP was measured at baseline (7 weeks of age) and then weekly until the end of either chronic study period.

Acute Renal Clearance Experiments

Nine rats from each 6-week feeding group and nine rats from each 12-week feeding group were subjected to renal clearance experiments at the end of their respective feeding periods as previously described [176]. Briefly, the right inguinal area was shaved, a small (< 2 cm) incision made, and femoral vessels isolated. The right femoral artery was cannulated with heparin-primed (100 U/ml) PE-50 polyethylene tubing connected to a pressure transducer (PowerLab data acquisition system; ADInstruments, Colorado Springs, CO) for continuous measurement of arterial pressure. The right femoral vein was catheterized with heparin-primed PE-50 tubing for infusion of solutions at 20 µl/min. An isotonic saline solution containing 6% albumin was infused via the venous line during surgery for arterial line and bladder catheter placement. After surgery, the infusion fluid was changed to isotonic saline containing 2% bovine serum albumin (BSA), 7.5% inulin (Inutest), and 1.5% PAH. The bladder was exposed via a suprapubic incision and catheterized with a PE-200 tube (with one end flanged) for gravimetric

urine collection. After a 15- to 20-minute stabilization period, a 30-minute clearance period was conducted to assess values of renal hemodynamic parameters. An arterial blood sample was collected at the end of the 30-minute clearance collection period for measurement of plasma inulin and PAH concentrations. Plasma inulin and PAH concentrations were measured colorimetrically to determine glomerular filtration rate (GFR) and renal plasma flow (RPF), respectively.

Electron Paramagnetic Resonance (EPR) Spectroscopy

Total ROS, superoxide ($O_2^{\bullet -}$), and peroxynitrite ($OONO^-$) production rates were measured in pieces of kidney cortex or medulla (chronic and 2-day feeding studies) and in liver and cerebral cortex (2-day feeding study) via EPR spectroscopy as previously published [194-199] and described in Chapter 2. In this EPR protocol, 'total ROS' represents all reactive oxygen species; however, the major sources trapped by the spin trap used are superoxide, hydrogen peroxide, and hydroxyl radical, with other species as minimal contributors. Briefly, tissue pieces were incubated at 37^{o}C with 6.6 μl of CMH (200 μM) for 30 minutes for ROS measurement; 1.5 μl of PEG-SOD (50 U/μl) for 30 minutes, then CMH for an additional 30 minutes for superoxide measurement; or 30 μl of CPH (500 μM) for 30 minutes for peroxynitrite measurement. Aliquots of incubated probe media were then taken in 50 μl disposable glass capillary tubes (Noxygen Science Transfer and Diagnostics, Elzach, Germany) for determination of total ROS, superoxide, or peroxynitrite production.

Measurement of Antioxidant Status

Catalase activity and total glutathione (GSH) levels were measured in kidney cortex and medulla (chronic and 2-day feeding studies) and also in liver and left ventricle (2-day feeding

study) using commercially available kits (Cayman Chemical, Ann Arbor, MI) according to manufacturer's instructions, as previously described [200].

Statistical Analyses

A two-way ANOVA (strain x diet) was used to analyze blood pressure, food consumption, body weight, physiological, biochemical, and EPR data at each time-point. Where significant main effects or interactions were found, individual planned comparisons were made using Student's t-tests for all other chronic feeding study data specifically to compare WC and WBB animals; WC and SC animals; and SC and SBB animals. T-tests were also used to compare results from SHR C and SHR BB groups for the 2-day feeding study. In all cases, $p \leq 0.05$ was accepted as the level of statistical significance.

Results

Chronic Feeding Studies

Consistent with past studies using similar dietary formulations [183, 188, 189, 201], weekly food consumption and body weight gain did not differ among any of the diet groups in the chronic feeding studies. Mean starting body weights in the WKY and SHR animals at baseline before assignment to groups were 138 ± 3 g and 142 ± 2 g, respectively; at the end of the 6 week study, the mean body weights were as follows: WC = 252 ± 9 g; WBB = 246 ± 3 g; SC = 250 ± 3 g; and SBB = 252 ± 3g. At the end of the 12 week study, mean body weights were as follows: WC = 346 ± 4 g; WBB = 342 ± 5 g; SC = 334 ± 6 g; and SBB = 333 ± 8 g.

Figure 4.1 presents the BP trends for each group of rats in both the 6-week and 12-week studies. Compared to SC rats, the mean arterial and systolic pressures of the SBB rats were significantly lower by the second week of the 6-week and 12-week studies, and remained significantly lower for the remainders of both chronic studies. Table 4.1 presents the

renoprotective effects of BB diet in SHRSP rats fed for 6 weeks or 12 weeks. Glomerular filtration rate and renal blood flow were higher, and renal vascular resistance was lower, in 6-week and 12-week SBB rats when compared to SC rats. There were no significant differences in renal hemodynamic or BP measures between WC or WBB animals. The decreases in total ROS, superoxide, and peroxynitrite seen with chronic BB feeding for 6 or 12 weeks appear in Table 4.2. For both 6- and 12-week studies, SBB rats exhibited significantly lower free radical production rates than SC rats.

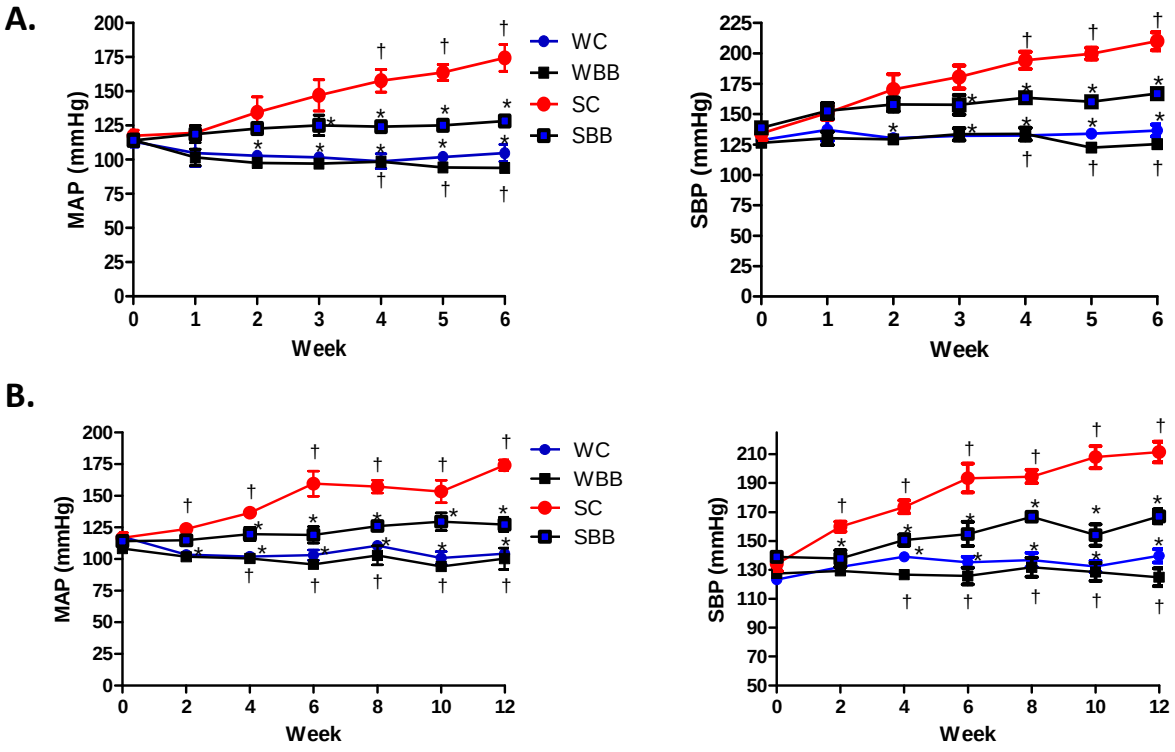

Figure 4.1. Blueberry-enriched diet delays the progression of hypertension. Mean arterial and systolic blood pressures were assessed in rats fed a corn diet or a blueberry-enriched diet for 6 weeks (A) or 12 weeks (B). * $p<0.05$ vs. SC; † $p<0.05$ vs. SBB.

Table 4.1. Renal hemodynamic indices in corn- or blueberry-fed rats after 6 weeks or 12 weeks of feeding.

		WC	WBB	SC	SBB
6 WEEKS	**GFR**	$0.95 \pm 0.05^{*}$	0.92 ± 0.05	$0.59 \pm 0.04^{\dagger}$	$0.97 \pm 0.07^{*}$
	RBF	$7.98 \pm 0.25^{*}$	8.42 ± 0.32	$5.96 \pm 0.35^{\dagger}$	$7.71 \pm 0.17^{*}$
	RVR	$13.33 \pm 0.70^{*}$	14.28 ± 1.24	$28.04 \pm 1.39^{\dagger}$	$13.34 \pm 0.63^{*}$
12 WEEKS	**GFR**	$0.90 \pm 0.06^{*}$	1.11 ± 0.09	$0.53 \pm 0.04^{\dagger}$	$1.02 \pm 0.07^{*}$
	RBF	$7.03 \pm 0.25^{*}$	8.42 ± 0.75	$3.62 \pm 0.22^{\dagger}$	$6.80 \pm 0.59^{*}$
	RVR	$15.98 \pm 1.14^{*}$	14.05 ± 1.41	$36.71 \pm 2.10^{\dagger}$	$15.49 \pm 1.22^{*}$

* $p<0.05$ vs. SC; † $p<0.05$ vs. SBB.

Table 4.2. Total ROS, superoxide, and peroxynitrite production rates as measured by EPR in tissues of corn- or blueberry-fed rats after 6 or 12 weeks of feeding.

	WC	WBB	SC	SBB
KIDNEY CORTEX				
Total ROS				
6 weeks	$0.067 \pm 0.012^{*}$	$0.099 \pm 0.005^{\$}$	$0.199 \pm 0.027^{\dagger}$	$0.069 \pm 0.011^{*}$
12 weeks	$0.115 \pm 0.013^{*}$	$0.112 \pm 0.009^{\$}$	$0.429 \pm 0.038^{\dagger}$	$0.195 \pm 0.026^{*}$
Superoxide				
6 weeks	$0.040 \pm 0.014^{*}$	0.028 ± 0.006	$0.136 \pm 0.026^{\dagger}$	$0.030 \pm 0.007^{*}$
12 weeks	$0.067 \pm 0.018^{*}$	0.063 ± 0.013	$0.165 \pm 0.013^{\dagger}$	$0.063 \pm 0.006^{*}$
Peroxynitrite				
6 weeks	$0.017 \pm 0.002^{*}$	$0.022 \pm 0.003^{\$}$	$0.053 \pm 0.011^{\dagger}$	$0.011 \pm 0.003^{*}$
12 weeks	$0.020 \pm 0.006^{*}$	0.028 ± 0.004	$0.111 \pm 0.021^{\dagger}$	$0.028 \pm 0.020^{*}$
KIDNEY MEDULLA				
Total ROS				
6 weeks	$0.077 \pm 0.011^{*}$	$0.087 \pm 0.007^{\$}$	$0.188 \pm 0.025^{\dagger}$	$0.118 \pm 0.010^{*}$
12 weeks	$0.157 \pm 0.014^{*}$	0.151 ± 0.018	$0.314 \pm 0.017^{\dagger}$	$0.170 \pm 0.017^{*}$
Superoxide				
6 weeks	$0.056 \pm 0.011^{*}$	0.051 ± 0.013	$0.122 \pm 0.009^{\dagger}$	$0.048 \pm 0.011^{*}$
12 weeks	$0.079 \pm 0.012^{*}$	0.075 ± 0.008	$0.164 \pm 0.017^{\dagger}$	$0.089 \pm 0.019^{*}$
Peroxynitrite				
6 weeks	$0.027 \pm 0.006^{*}$	0.021 ± 0.005	$0.050 \pm 0.005^{\dagger}$	$0.025 \pm 0.005^{*}$
12 weeks	$0.052 \pm 0.017^{*}$	$0.065 \pm 0.005^{\$}$	$0.128 \pm 0.009^{\dagger}$	$0.031 \pm 0.007^{*}$

* $p<0.05$ vs. SC; † $p<0.05$ vs. SBB.

The increases in cortical and medullary catalase and glutathione activities noted with chronic BB-enriched diet feedings appear in Figures 4.2 and 4.3, respectively. For both chronic feeding studies, SBB rats exhibited significantly higher catalase and glutathione activities than SC rats. There were no differences in antioxidant activities between WC and WBB rats at the conclusion of the 6- or 12-week studies.

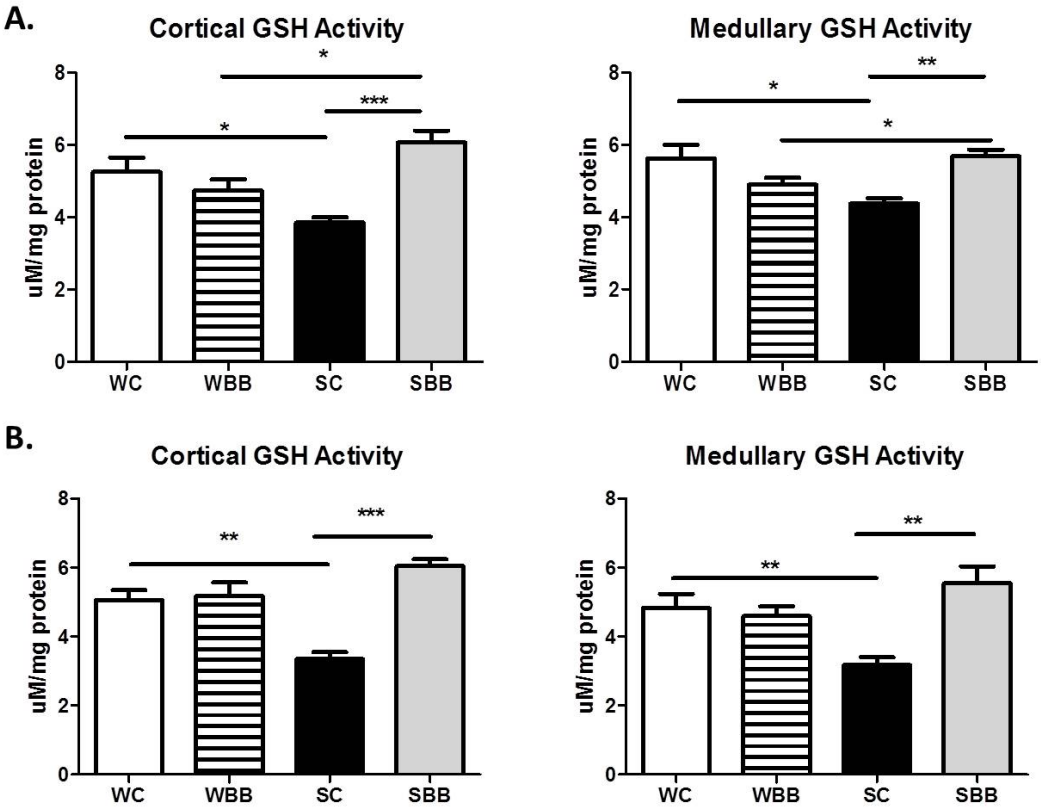

Figure 4.2. Blueberry-enriched diet improves glutathione activity in hypertensive rats. Glutathione activities were assessed in renal cortical and medullary tissues of rats fed a corn diet or a blueberry-enriched diet for 6 weeks (A) or 12 weeks (B). $^{*}p<0.05$; $^{**}p< 0.01$; $^{***}p<0.001$.

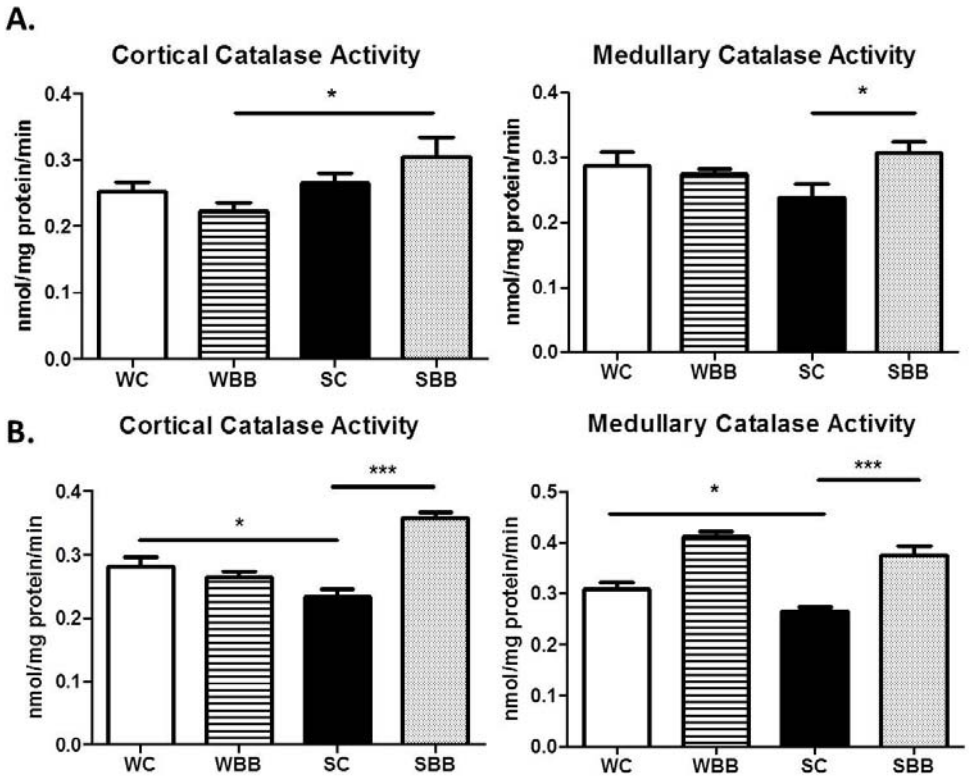

Figure 4.3. Blueberry-enriched diet improves catalase activity in hypertensive rats. Catalase activities were assessed in renal cortical and medullary tissues of rats fed a corn diet or a blueberry-enriched diet for 6 weeks (A) or 12 weeks (B). $^{*}p<0.05$; $^{**}p< 0.01$; $^{***}p<0.001$.

Short-Term Feeding Studies

No significant differences were found in body weights between corn- or BB-diet fed animals in the 2-day feeding study. As noted in Table 4.3, significant increases in production rates of total ROS, superoxide, and peroxynitrite were observed in cerebral cortex, liver, kidney cortex, and kidney medulla of rats fed a BB diet for 2 days compared to those on corn diet. Table 4.4 depicts the catalase and glutathione activities recorded in heart, liver, and kidney tissues of 2-day BB-fed SHRSP when compared to corn diet-fed SHRSP. In the left ventricle and kidney cortex of SHRSP fed BB-diet for 2 days, catalase activities were increased when compared to those of SHRSP fed a corn diet for 2 days. However, total GSH levels were lower

in the left ventricle of 2 day BB-fed rats when compared to 2-day corn diet-fed rats, with no significant changes in GSH levels noted in other tissues assayed.

Table 4.3. Total ROS, superoxide, and peroxynitrite production rates as measured by EPR in tissues of corn- or blueberry-fed rats after 2 days of feeding.

	Corn (n= 7)	Blueberry (n= 7)	P
CEREBRAL CORTEX			
Total ROS	0.126 ± 0.008	0.156 ± 0.015	0.0417
Superoxide	0.024 ± 0.003	0.039 ± 0.005	0.0087
Peroxynitrite	0.005 ± 0.001	0.022 ± 0.003	0.0004
LIVER			
Total ROS	0.371 ± 0.026	0.530 ± 0.057	0.0129
Superoxide	0.150 ± 0.007	0.246 ± 0.023	0.0046
Peroxynitrite	0.014 ± 0.002	0.042 ± 0.007	0.0069
KIDNEY CORTEX			
Total ROS	0.140 ± 0.013	0.210 ± 0.022	0.0289
Superoxide	0.041 ± 0.005	0.063 ± 0.004	0.0275
Peroxynitrite	0.015 ± 0.002	0.034 ± 0.004	0.0044
KIDNEY MEDULLA			
Total ROS	0.146 ± 0.011	0.189 ± 0.019	0.0136
Superoxide	0.060 ± 0.011	0.106 ± 0.025	0.0258
Peroxynitrite	0.035 ± 0.006	0.075 ± 0.011	0.0158

Table 4.4. Catalase and total glutathione (GSH) levels as measured by colorimetric assays in tissues of corn- or blueberry-fed rats after 2 days of feeding.

	Corn (n= 8-10)	Blueberry (n= 8-10)	P
LEFT VENTRICLE			
Catalase	0.293 ± 0.019	0.373 ± 0.022	0.0285
Total GSH	2.68 ± 0.243	1.89 ± 0.213	0.0495
LIVER			
Catalase	0.287 ± 0.011	0.276 ± 0.016	n.s.
Total GSH	1.91 ± 0.159	1.85 ± 0.065	n.s.
KIDNEY CORTEX			
Catalase	0.246 ± 0.012	0.306 ± 0.020	0.0331
Total GSH	2.11 ± 0.187	2.23 ± 0.091	n.s.
KIDNEY MEDULLA			
Catalase	0.325 ± 0.010	0.310 ± 0.028	n.s.
Total GSH	2.23 ± 0.207	2.17 ± 0.065	n.s.

Discussion

Primary (essential) hypertension remains a major cause of morbidity and mortality in Western society, and continues to be a leading cause of heart and kidney diseases [202]. The cause(s) of primary hypertension remain elusive; however, oxidative stress and proinflammatory cytokine production are known contributors [203, 204]. Nephropathy resulting from hypertension is the second leading cause of end-stage renal disease in the United States [202]; therefore, the most effective way to avoid the development of hypertensive nephropathy is to prevent hypertension or to delay its progression. In many cases, hypertension can be attenuated with pharmacological treatments including, but not limited to: diuretics, beta receptor antagonists, angiotensin converting enzyme antagonists, and angiotensin II receptor antagonists; however, these commonly used anti-hypertensives can also have undesirable side effects. Therefore, it is valuable to consider natural products, such as foods, as potentially therapeutic sources of antioxidants for a variety of conditions.

Thus far, a variety of pharmacotherapies have proven successful in decreasing renal damage in hypertensive animals; however, the possible benefits of dietary interventions have only recently come into focus. BB-enriched diets have been shown to decrease renal nitrite content, protect the myocardium from ischemia, and correct neurological deficits in rats [183, 185-189]. In the present study, we show for the first time that regular dietary supplementation with blueberries in SHRSP rats preserves renal hemodynamics and prevents oxidative stress in the kidney. We also demonstrate that BB may act via a hormetic mechanism in preventing oxidative stress in the SHRSP rat.

After 6 weeks and 12 weeks of BB feeding, GFR and RBF measures were higher, estimated RVR was lower, renal free radical production was attenuated, and renal antioxidant

levels were preserved in BB-fed SHRSP when compared to those of SHRSP maintained on a corn diet. The results of our chronic feeding experiments also demonstrate that total ROS, superoxide, and peroxynitrite production rates were significantly lower and antioxidant activities were significantly higher in BB-fed SHRSP than in corn-fed SHRSP. These results clearly demonstrate a protective antioxidant effect of BB feeding. The imbalance between superoxide production and NO production in the kidney is a primary contributor to renal oxidative stress and salt-sensitive hypertension [205, 206]. Oxidative stress is further enhanced in the kidneys of SHRSP that are salt-loaded (as were the SHRSP in this study) [206, 207]. We demonstrate here that the BB diet protected against oxidative renal damage by attenuating free radical production and preserving antioxidant status, and thereby improving BP and renal hemodynamics.

A possible mechanism for this renoprotection may be the scavenging of superoxide in kidney tissues, which has been shown to lower BP in various models of hypertension [206, 208]. BB are known scavengers of ROS/RNS, including superoxide, *in vitro* [209]. In further support of a renal superoxide scavenging mechanism, we found that the cortical and medullary production rates of peroxynitrite in BB-fed rats from both 6- and 12-week time-points were significantly lower compared to rats fed the corn diet. Nitric oxide reacts readily with superoxide to form the highly cytotoxic peroxynitrite radical; therefore, one explanation for the decline in peroxynitrite production in BB-fed animals is an increase in nitric oxide, which can foster the removal of superoxide. Further study is needed to determine conclusively whether this effect is responsible for the renoprotection afforded by chronic BB feeding.

These chronic feeding studies were not intended to analyze specific signaling pathways responsible for preservation of renal hemodynamics and/or reduction of oxidative stress in the kidneys of hypertensive animals on a BB-enriched diet, but rather as a proof of concept. One

assertion that can be made on the basis of these findings is that alterations in signaling were likely associated with decreases in ROS/RNS production and improvements in ROS/RNS scavenging, which likely affected inflammatory status. Although we did not examine inflammation in these studies *per se*, we can infer, based on our previous research, that this effect was mediated in part through the inflammatory nuclear factor kappa B (NF-κB) pathway, which can both induce and respond to oxidative stress [193]. Inferences can also be made on the basis of previous studies. BB extract can significantly inhibit the lipopolysacharide-induced inflammatory response in brain microglia by downregulating inducible nitric oxide synthase; therefore, BB extract may indeed inhibit an early step of the inflammatory stress pathway transcribed by NF-κB [187]. In the same model of mouse microglial cells, BB extract also inhibited the expression of cyclooxygenase-2, which is known to be associated with the NF-κB-regulated proinflammatory cytokines, interleukin-1β and tumor necrosis factor-α [187]. Extrapolating from these findings, it is plausible to suggest that some of the same oxidative/inflammatory mechanisms may be at work in the hyptertensive kidney.

Our results from the 2-day feeding study indicate that a hormetic effect of BB may indeed exist in the prevention of hypertension-induced renal hemodynamic alterations. The 'xenohormesis' hypothesis proposes that animal species have evolved the ability to use chemical cues from plant species to mount a preemptive defense response that increases its chances of survival [191, 210]. Polyphenols, among other phytochemicals, are thought to exert many of their beneficial effects via hormetic mechanisms [210]. In contrast to the clear evidence of reduced ROS/RNS production in the long-term studies, results from the 2-day feeding study indicated significant increases in total ROS, superoxide, and peroxynitrite production in kidney, brain, and liver tissues of BB-fed rats when compared to corn-fed rats. As a response to this

situation, increased catalase activities were found with 2-day BB feeding, but only in kidney cortex and left ventricular tissues. Overall, the EPR and antioxidant assay results suggest that, in the case of BB feeding, an initial oxidative stimulus is produced, which is presumably required for the antioxidant defense to be activated, thereby supporting the assertion that a hormetic effect is involved in the protection afforded by BB *in vivo*. Since we evaluated hormetic responses only at the 2-day time-point of BB exposure, further analysis is required to document in detail the kinetics of ROS production and antioxidant responses.

In summary, our experiments examining rats chronically maintained on a BB-enriched diet for 6 or 12 weeks found preservation of renal hemodynamics and decreased blood pressure. Further, the BB diet decreased ROS/RNS production and preserved antioxidant status in the kidney cortex and medulla of chronically fed hypertensive rats. The beneficial effects of the BB diet may be due to a hormetic effect, as evidenced by our results from the 2-day feeding experiment, where ROS/RNS production was increased in all tissues of BB-fed animals, while response of the antioxidant system was in a state of flux, with some systems elevated at that time-point and others unresponsive. This is the first demonstration, to our knowledge, of the effectiveness of a readily available natural product in an acceptable, consumable quantity to significantly attenuate hypertension-induced renal functional alterations.

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CHAPTER 5

A BLUEBERRY-ENRICHED DIET DELAYS RENAL FAILURE IN THE STROKE-PRONE SPONTANEOUSLY HYPERTENSIVE RAT

Introduction

Nephropathy is a leading cause of morbidity and mortality in hypertensive patients [211]. Many preferred pharmacological treatments for hypertension are renoprotective; however, the clinical courses of hypertension and its associated target organ damage remain progressive. Therefore, a continuous need for new therapeutic approaches exists. Oxidative stress and inflammation are common features of hypertensive nephropathy and also contribute to its progression [212-215]. Oxidative stress in hypertension is caused by a combination of increased reactive oxygen species (ROS) production and impaired antioxidant defenses [215-220]. Increased ROS production can modify various structural and functional molecules and activate redox-sensitive transcription factors, thereby leading to tissue injury and promoting inflammation, fibrosis, and further ROS production [221, 222].

The beneficial effects of antioxidant-rich diets in attenuating hypertension and improving endothelial function are well-established in animals and humans [223-227]. The Dietary Approaches to Stop Hypertension study, which implemented a fruit- and vegetable-rich diet in hypertensive patients, resulted in significant blood pressure reductions in those patients [228]. Further, epidemiological evidence indicates that dietary antioxidants may delay the onset of hypertension in some individuals [229, 230]. Previous studies have shown that polyphenol-rich fruits and vegetables are beneficial in both delaying and treating hypertension [231, 232]. The protective effects observed in these studies may be due to the antioxidant and anti-inflammatory properties attributed to polyphenolic compounds. Since inflammation and oxidative stress are

associated with hypertension-related renal injury, it is plausible to suggest that consumption of foods high in antioxidants and anti-inflammatory agents may delay or prevent its development.

The oxygen radical absorbance capacity (ORAC) assay is a widely accepted method for measuring the total antioxidant capacities of fruits and vegetables [233, 234]. The ORAC value for blueberries is one of the highest among all fruits and vegetables [235, 236]; consequently, blueberries have been shown to protect against inflammation [237, 238], oxidative stress [237, 239, 240], and chronic diseases [241]. Recent research has highlighted the promise of blueberries in lowering blood pressure and tissue nitrites in spontaneously hypertensive stroke-prone (SHRSP) rats [227], in addition to protecting the heart from ischemic damage [242] and improving cognition and other neurological parameters [239, 243]. However, the direct effects of a blueberry-enriched diet on renal hemodynamics and renal oxidative stress have not yet been examined; this was the focus of the present study. Here, we hypothesized that supplementing a stroke-permissive rodent diet with 2% blueberry (BB) extract for 10 weeks would preserve renal hemodynamics and beneficially alter redox status in the SHRSP kidney.

Materials and Methods

All experimental procedures were in compliance with all applicable principles set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Publication No.. 85-23, revised 1996). This study was approved by the Institutional Animal Care and Use Committee of the Louisiana State University School of Veterinary Medicine.

Chemicals and Drugs

Inulin (polyfructosan-S; Inutest) was obtained from Fresenius-Kabi (Graz, Austria) and para-aminohippurate (PAH) was obtained from Merck, Sharp & Dohme (West Point, PA). The spin probes 1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine (CMH), 1-hydroxy-

3-carboxypyrrolidine (CPH), and 1-hydroxy-4-phosphono-oxy-2, 2, 6, 6-tetramethylpiperidine (PPH); the metal chelators defferoxamine (DF) and diethyldithiocarbamate (DETC); and Krebs-HEPES buffer (KHB) were obtained from Noxygen Science Transfer and Diagnostics (Elzach, Germany). Polyethylene glycol-conjugated superoxide dismutase (PEG-SOD) and thiobutabarbital (Inactin) were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals and reagents used were of analytical grade and were purchased from Sigma-Aldrich unless otherwise specified.

Animals

Eighteen male spontaneously hypertensive stroke prone rats (SHRSP) and six normotensive Wistar-Kyoto (WKY) control rats were used in this study. Animals were 21 days old at the start of the experiment. All animals were provided food and water *ad libitum*, and maintained in a temperature (23 ± 2°C)- and light (12 hour light/dark cycle)-controlled environment. Prior to the start of the experiment, rats were randomly assigned to one of four diet groups. Rats were maintained on diets for ten consecutive weeks: WKY Japanese stroke permissive (JSP) diet, SHRSP regular diet, SHRSP JSP diet, or SHRSP JSP BB-supplemented (JSP BB) diet. The JSP diet is low in protein and potassium and high in sodium, and has been demonstrated to accelerate organ pathology in the SHRSP rat [244-246]. Rats were fed their respective diets for 10 weeks, from date of weaning (age 3 weeks) until the age of 14 weeks. All animals were provided 1% NaCl in tap water for the duration of the study; when combined with JSP diet, this has previously been demonstrated to induce early renal damage in the SHRSP model [244-247].

Diets

Blueberry preparations were a generous gift from Drs. Barbara Shukitt-Hale and James A. Joseph (Tufts University, Boston, MA). Blueberries were prepared as previously described [248, 249]. Briefly, blueberries were homogenized and centrifuged, and the supernatant was then frozen, crushed, and lyophilized. Freeze-dried blueberry preparations were then shipped to Harlan Teklad (Madison, WI), where they were combined with the JSP control diet at 20 grams per kilogram diet (2% w/w). Macronutrient compositions of both diets appear in Table 5.1.

Table 5.1. Macronutrient contents of control and blueberry-enriched diets.

% by weight	**Control Diet**	**BB-Enriched Diet**
Protein	16.4	16.0
Carbohydrate	48.5	53.5
Fat	4.0	3.5
Kilocalories/gram	3.0	3.1
% kcal from:	**Control Diet**	**BB-Enriched Diet**
Protein	22	21
Carbohydrate	66	69
Fat	12	10

Animal Monitoring and Surgical Procedures

After ten weeks on the control, JSP, or JSP BB diets, each rat was anesthetized with Inactin (thiobutabarbital; 100 mg/kg), and then underwent surgery for catheterization of the femoral artery, femoral vein, and bladder; acute renal clearance experiments were then performed as previously described [222]. Arterial pressures were obtained for each animal during surgery, via a fluid-filled pressure transducer. After completion of renal clearance studies, rats were euthanized by Inactin overdose. The chest was then cut to expose the heart, and blood was removed via cardiac puncture. Kidney tissues and plasma were collected for later analyses.

Glomerular Injury Scoring and Histopathological Evaluation

Trichrome-stained kidney sections (3 μm) from rats were examined by a veterinary pathologist who was blinded to the experimental conditions. One hundred glomeruli from each section were scored. A semi-quantitative glomerular lesion scoring method was used, which was based upon previously published methods for glomerulosclerosis scoring [250] and expanded to include the following criteria: tubular epithelial metaplasia of Bowman's capsule, glomerulosclerosis, mesangial proliferation, and glomerular capillary basement membrane thickening. A detailed explanation of this scoring method appears in Chapter 3.

NF-κB p65 DNA Binding Activity Assay

The binding of NF-κB p65 to its DNA consensus binding site was measured in renal cortical nuclear extracts with a TransAM NF-κB p65 ELISA Kit (Active Motif; Carlsbad, CA), according to manufacturer's instructions, as previously described in Chapter 3.

Western Blotting

Protein expression in renal cortical nuclear and cytoplasmic extracts was analyzed by western blot as previously described [222, 251], using anti-p65, anti-SIRT1, anti-JNK, anti-phospho-JNK, anti-histone H1 and anti-GAPDH antibodies. Densitometry analyses were performed with Image J software. Three blots each were performed for the SIRT1 antibody; each membrane was then reblotted with anti-histone H1 antibody (nuclear loading control). Three blots each were performed for JNK and phospho-JNK antibodies; each membrane was then reblotted with anti-GAPDH antibody (cytoplasmic loading control).

Cortical Tissue EPR Measurements

Cortical total ROS, superoxide, and peroxynitrite production rates were measured using EPR spectroscopy as previously described [222, 252]. In this EPR protocol, 'total ROS'

represents all reactive oxygen species; however, the major sources trapped by the spin trap used are superoxide, hydrogen peroxide, and hydroxyl radical, with other species as minimal contributors.

Measurement of Nitrate/Nitrite Levels

Nitrate/nitrite levels (indirect indicator of NO activity) were measured in kidney cortex using a commercially available kit, according to manufacturer's instructions (Cayman Chemical, Ann Arbor, MI), as previously described [252].

Measurement of Catalase and Glutathione Levels

Cortical antioxidant status was assessed by measurement of catalase and total glutathione levels in kidney cortex using commercially available kits, according to manufacturer's instructions (Cayman Chemical, Ann Arbor, MI), as previously described [222, 253].

Statistical Analyses

All data are presented as means ± SEMs. A two-way ANOVA (strain x diet) was used to analyze blood pressure, food consumption, and body weight data. Where significant main effects or interactions were found, individual planned comparisons were made using Student's t-tests for all other parameters measured, specifically to compare WKY and SHRSP animals; SHRSP and JSP animals; and JSP and JSP BB animals. In all cases, $p \leq 0.05$ was accepted as the level of statistical significance.

Results

In a pilot study with eight SHRSP rats fed the JSP diet, only three rats survived until 10 weeks of age (Figure 5.1). In the WKY, SHR, and JSP BB groups, all animals survived until study end. These results indicate that BB supplementation prolongs survival in the SHRSP model of hypertensive nephropathy. At study end, JSP animals had significantly lower body

weights than SHRSP and JSPBB animals. No differences in body weights were noted between WKY and SHRSP animals (Table 5.2).

Mean arterial pressures and systolic blood pressures were significantly higher in the JSP group than in the JSP BB or SHRSP groups at study end (Table 5.2). Interestingly, WKY animals fed the JSP diet did not exhibit elevations in blood pressure. Significantly higher GFR and RBF values and significantly lower RVR and urine albumin values were seen in JSP BB animals when compared to the JSP group; these results suggest that BB supplementation preserves renal hemodynamics in the SHRSP rat fed a stroke-permissive diet. Further, urinary albumin levels were significantly higher in the JSP group than in the JSP BB or SHRSP groups at study end; these results further demonstrate the preservation of renal function in BB-fed rats.

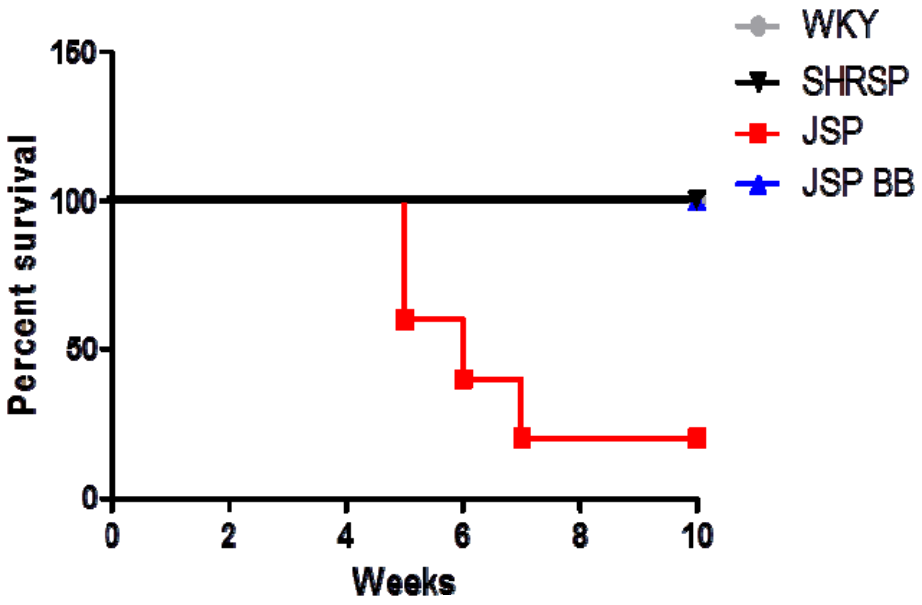

Figure 5.1. A survival curve was constructed to determine the effects of a blueberry-enriched diet on survival in stroke-prone SHR. JSP animals began to die at 5 weeks of age; only 3 animals remained at the conclusion of the study. All WKY and all JSP BB animals survived the duration of the 10 week study.

Table 5.2. Renal hemodynamic parameters obtained from all animals at study conclusion.

	WKY	SHRSP	JSP	JSP BB
BW (g)	322±8.95	309±5.81*	281±7.14$^{\dagger\#}$	301±1.65*
MAP (mmHg)	92.7±2.61†	131.1±2.21$^{\$}$	153.9±2.63$^{\#}$	126.2±7.42*
SBP (mmHg)	120.8±3.71†	158.6±5.59$^{\$}$	182.6±7.69$^{\#}$	143.9±6.84*
GFR (ml/min/g KW)	0.99±0.10†	0.65±0.04$^{*\$}$	0.42±0.04$^{\dagger\#}$	0.80±0.04*
RBF (ml/min/g KW)	6.94±0.62†	5.81±0.46$^{*\$}$	3.07±0.21$^{\dagger\#}$	5.69±0.25*
RVR(mmHg/ml/min/g KW)	13.64±1.16†	24.09±3.53$^{*\$}$	43.74±1.68$^{\dagger\#}$	29.27±2.85*
Urine Albumin (mg/dl)	41.8±3.79†	53.3±1.65$^{\$}$	65.7±2.67$^{\dagger\#}$	49.9±2.21*

$^{\$}$ $p<0.05$ vs. WKY; † $p<0.05$ vs. SHRSP; * $p<0.05$ vs. JSP; # $p<0.05$ vs. JSP BB.

Rats from the JSP group had significantly higher glomerular lesion scores than rats from SHRSP and JSP BB groups at the completion of the study (Figure 5.2A). There were similar histopathologic changes in all hypertensive rats; however, the prevalence and severity of changes differed between groups (Figures 5.2B-E). In the end-stage kidneys seen in the JSP rats, profound arterial sclerosis and frequent arterial thrombosis were present. JSP BB rats also had arteriosclerosis but had less frequent thromboses. As expected, given the circulatory compromise, the degree of tubular degeneration and dilation in the JSP rats was much more severe than in JSP BB rats. Although changes in glomeruli were similar in JSP and JSP BB groups, greater protein accumulation in Bowman's space and in tubules suggests that the JSP rats had more severe glomerular leakage/damage; these findings are further supported by the significantly higher urinary albumin levels in the JSP animals and the decrease in urinary albumin levels seen with BB feeding.

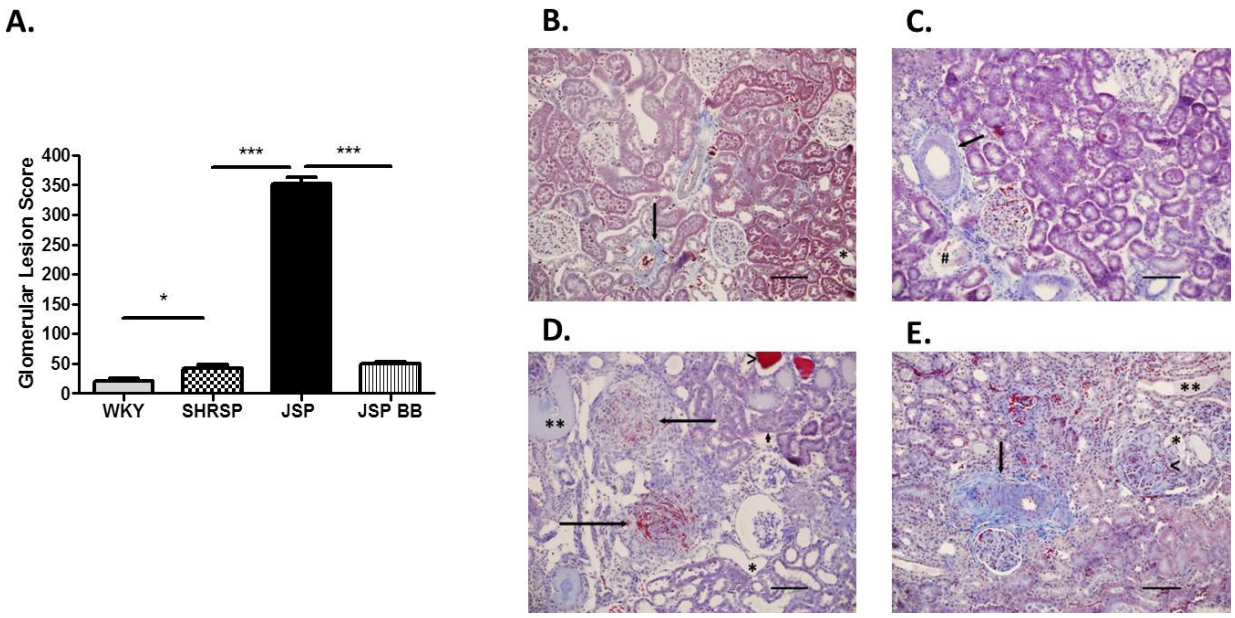

Figure 5.2. A) Glomerular scores for rats in all experimental groups. B) WKY rat kidneys had mild periarterial fibrosis and minimal smooth muscle hyperplasia (long arrow). Mild interstitial fibrosis was also present (in the right upper corner of the image) and there was rare tubular degeneration (*). C) SHRSP kidneys exhibited moderate periarterial fibrosis (long arrow) and mild smooth muscle hyperplasia. Occasional senescent glomeruli (#) were also present. D) JSP rat kidneys exhibited medium-sized arteries with hypertrophied tunica media and moderate periarterial fibrosis, which were often thrombosed with remodeling and recanalization (long arrow). The JSP kidneys also had more frequent tubular degeneration (*) and areas of tubular regeneration (short arrow – tubules lined by increased numbers of plump epithelial cells). They also had more frequent tubular ectasia (**) with eosinophilic to lightly basophilic proteinaceous casts. Occasional hemoglobin casts within degenerate tubules were also seen in this group (>). E) In JSP BB rat kidneys, medium-sized arteries typically had hypertrophied tunica media with moderate periarterial fibrosis (black arrow), occasional mildly dilated tubules lined by flattened tubular epithelium (* tubular degeneration), occasional glomeruli with mild glomerular sclerosis/fibrosis (<), and occasional dilated tubules with flattened epithelium and lightly eosinophilic proteinaceous casts (**). Scale bars = 100 μm.

Protein expression levels of collagen III and TGF-β were assessed in animals from all experimental groups. There were no significant differences in collagen III or TGF-β levels between WKY and SHRSP rats; however, levels of both proteins were significantly higher in JSP rats when compared to SHRSP rats. JSP BB rats had significantly lower expression of both proteins, indicating a decrease in profibrotic molecules in the kidneys of these animals.

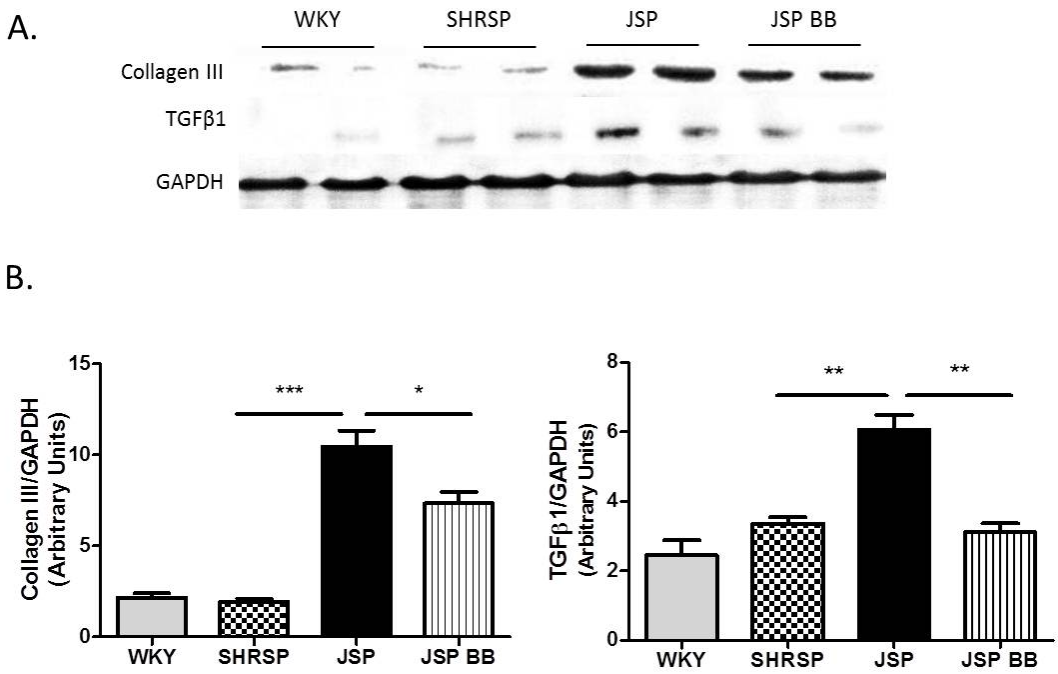

Figure 5.3. A) Representative western blots for collagen III and TGF-β in animals from all experimental groups. B) Densitometric analyses of western blots for collagen III and TGF-β. * $p<0.05$; ** $p<0.01$; ***$p<0.001$.

We measured total ROS, superoxide, and peroxynitrite production rates using EPR spectroscopy in cortical tissues from rats in all experimental groups. Production rates of all species measured were significantly lower in tissues of WKY rats when compared to SHRSP rats, in SHRSP rats when compared to JSP rats, and in JSP BB rats when compared to JSP rats (Table 5.3).

Catalase and total glutathione levels were measured in renal cortical tissues from animals from all experimental groups. We found significantly lower catalase and glutathione levels in SHRSP animals when compared to WKY animals, and in JSP animals when compared to SHRSP or JSP BB animals (Table 5.3)

Table 5.3. Free radical production rates and antioxidant activities obtained from cortical tissues at study conclusion.

	WKY	SHRSP	JSP	JSP BB
Total ROS	0.094±0.019†	0.174±0.021$^{*\$}$	0.414±0.085$^{\dagger\#}$	0.069±0.007*
Superoxide	0.032±0.008†	0.066±0.006$^{*\$}$	0.119±0.015$^{\dagger\#}$	0.023±0.009*
Peroxynitrite	0.026±0.005†	0.050±0.002$^{*\$}$	0.089±0.013$^{\dagger\#}$	0.025±0.005*
Catalase	0.99±0.09†	0.57±0.05$^{\$}$	0.54±0.04$^{\#}$	0.79±0.08*
Total GSH	6.98±0.83†	4.56±0.54$^{*\$}$	2.71±0.35$^{\dagger\#}$	6.47±0.61*

$^{\$}$ p<0.05 vs. WKY; † p<0.05 vs. SHRSP; * p<0.05 vs. JSP; # p<0.05 vs. JSP BB.

NF-κB p65 DNA binding activity was measured in cortical nuclear extracts from animals from all experimental groups. There was no difference in p65 DNA binding activity levels between WKY and SHRSP groups. A significant increase was seen in p65 DNA binding activity in the JSP group when compared to the SHRSP group; this increase was prevented with BB feeding (Figure 5.4).

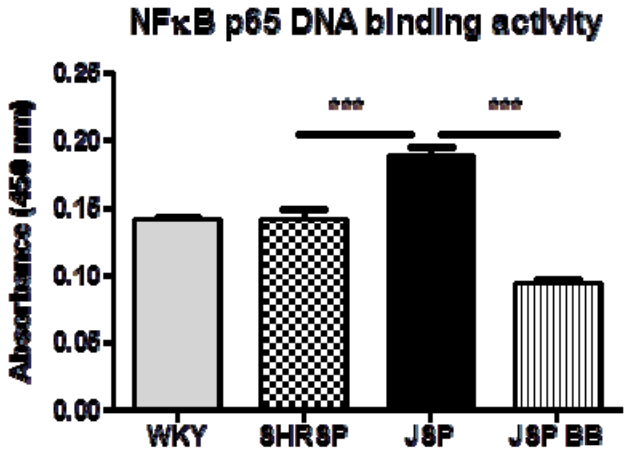

Figure 5.4. NF-κB p65 DNA binding activity measurements for cortical tissues from each experimental group. The p65 DNA binding activity of JSP tissues was greater than that of SHRSP, and BB feeding attenuated this increase. ***p<0.001.

Interestingly, total JNK expression only differed between SHRSP and JSP rats, while increased pJNK expression was seen in SHRSP rats when compared to WKY rats. A further

increase in pJNK expression was seen in JSP rats when compared to SHRSP rats; this increase was alleviated in the JSP BB rats. A significant decrease in SIRT1 expression was observed in JSP rats when compared to SHRSP rats, while this decrease was attenuated in JSP BB rats.

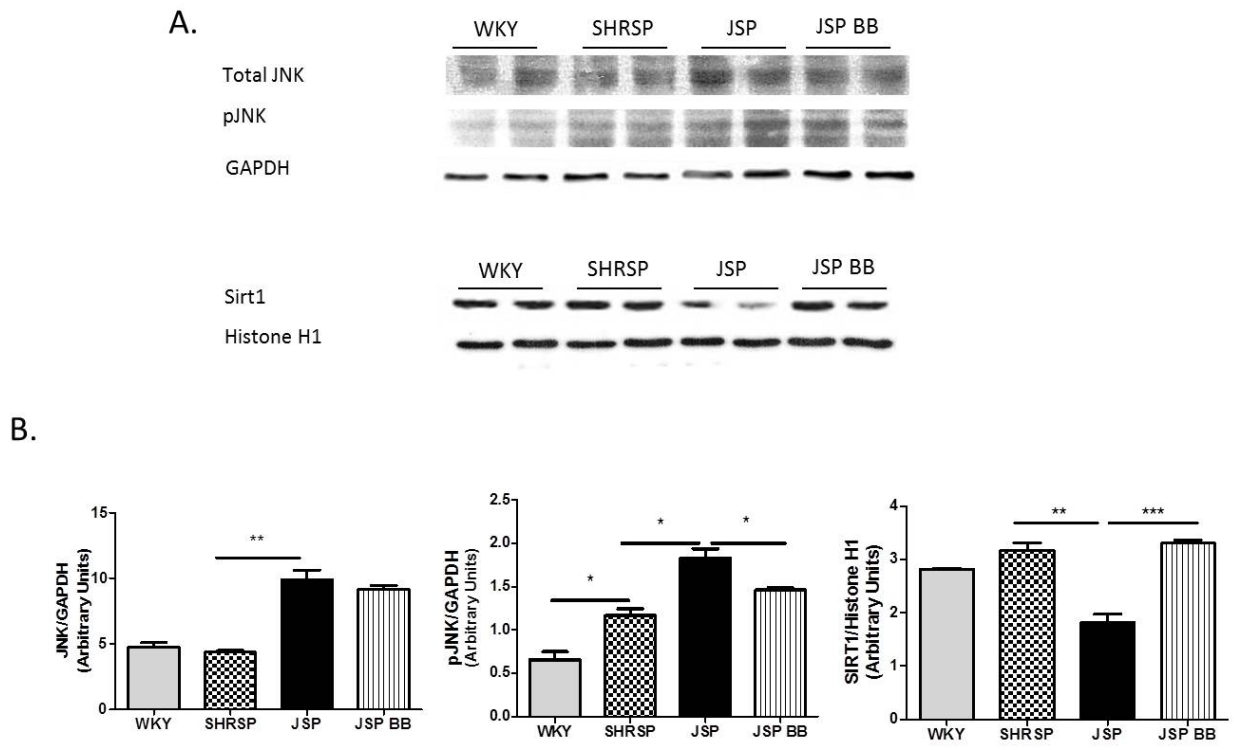

Figure 5.5. A) Representative western blots showing protein expression for total JNK, phosphorylated JNK, and SIRT1. GAPDH was used as the cytoplasmic loading control and histone H1 was used as the nuclear loading control. B) Densitometric analyses of total JNK, phospho JNK, and SIRT1 expression levels. * $p<0.05$; ** $p<0.01$; ***$p<0.001$.

Discussion

This study is the first to examine the beneficial effects of BB supplementation on renal structure and function in a model of severe hypertensive nephropathy. We hypothesized that supplementation of a stroke-permissive diet with 2% lyophilized blueberry would result in preservation of renal function and decreased oxidative stress in the salt-loaded SHRSP. When

fed a BB-enriched stroke-permissive diet, salt-loaded SHRSP demonstrated a preservation of renal hemodynamics, decreased urinary albumin loss, and a significant decrease in ROS/RNS production. Preservation of these parameters was accompanied by decreases in blood pressure and inflammation.

Renal damage is a major contributor to the development of hypertension in rats [254-257]. An imbalance between renal superoxide and nitric oxide production is a primary determinant of renal oxidative stress, leading to salt-sensitive hypertension [255, 258]. Further, oxidative stress is enhanced in the kidneys of SHRSP that are salt-loaded [255, 256], and becomes even more severe with the addition of a stroke-permissive diet, as was done in this study. In JSP rats, albuminuria was evident, along with significant declines in renal hemodynamic parameters and increased severity of renal lesions, indicating severe renal damage. Blueberry supplementation led to decreased urine albumin, preservation of renal hemodynamics, and decreased severity of renal pathology, suggesting that BB feeding can mitigate renal damage.

One possible mechanism for the protection seen with BB feeding may be scavenging of renal superoxide, which can significantly reduce BP in various animal models of salt-sensitive hypertension [240, 255, 256, 259]. Blueberries are potent scavengers of superoxide and other ROS *in vitro* [260, 261] and have been shown to reduce renal nitrites. Other antioxidants lower BP in SHR by diminishing renal damage and proteinuria [259, 262, 263]. Studies have also demonstrated that blueberry extract can inhibit NF-κB translocation and subsequently suppress the promoter activities of genes involved in inflammation in microglial cells, including iNOS and COX2 [264]. BB-enriched diets have also been shown to down-regulate the signaling cascades of several MAP kinases known to respond to oxidative stress, including that of JNK [265, 266].

The reduction in renal ROS/RNS production found in the JSP BB rats, along with improved activity of catalase and GSH, suggests an overall improvement in redox status in these animals, and implies an antioxidant effect of BB in the kidney. Similar effects have been found with the use of pharmacological antioxidants in hypertensive rats [267]. Also in agreement with those studies, we found a decrease in blood pressure elevation and improved renal hemodynamics in BB-fed rats, which can be attributed to decreased oxidative stress. Further, we found decreases in phosphorylated JNK, a redox-sensitive signaling molecule, and in activation of the redox-sensitive transcription factor, NF-κB.

We and others have demonstrated that increased ROS/RNS production can increase renal NF-κB activation in the hypertensive condition, thereby promoting the inflammatory response [213, 222, 268]. In the present study, we show an increase in NF-κB activation in kidney tissues of SHRSP rats compared to WKY rats, with a further increase in NF-κB activation in JSP kidneys compared to SHRSP kidneys. We also demonstrate that NF-κB activation is attenuated in kidneys of JSP rats fed a BB-enriched diet. Further, the decrease seen in NF-κB activation in the JSP BB animals was associated with decreased expression of phosphorylated JNK, a MAP kinase known to be up regulated in the kidneys of hypertensive rats [269, 270]. Historically, JNK has been shown to act through the transcription factor, activator protein-1 (AP-1). Recently, however, Cuhlmann *et al* reported a JNK-dependent activation of NF-κB in mouse arteries in response to disturbed blood flow, which led to arterial inflammation [271]. It is possible that this mechanism may be at work in the kidney, given our results demonstrating increased pJNK expression in association with increased NF-κB activation. Also, nuclear expression of the oxidative stress-responsive histone deacetylase SIRT1 was increased in the JSP

BB rats; this finding also supports the decrease seen in NF-κB activity, since SIRT1 is known to deacetylate p65 at lysine 310, thereby inhibiting its transactivation potential [272].

In a study by Nasrin and colleagues, JNK1 was recently shown to phosphorylate SIRT1 in kidney epithelial cells in response to hydrogen peroxide treatment, leading to an increase in the enzymatic activity of SIRT1 [273]. In contrast to those results, we found that the increase in JNK expression expected in JSP rats was associated with decreased SIRT1 nuclear expression in the kidney. There is one explanation for the differences in these results. The cellular hydrogen peroxide treatment in the study by Nasrin et al was of short duration (1 hour), with contrasting results found with 6 hours of treatment. The authors proposed that prolonged exposure to oxidative stress may lead to DNA damage and depletion of cellular NAD+ (a SIRT1 co-substrate), thereby leading to decreased SIRT1 activity, while acute exposure to low levels of oxidative stress may lead to increased SIRT1 activity in an initial attempt to promote cell survival. In our study, severe oxidative stress was observed in kidney tissues of JSP rats, thus, it is possible that this chronic oxidative stress did indeed deplete NAD+, causing a down-regulation of SIRT1. Further confirmatory studies are needed to verify this course of events.

Interestingly, SIRT1 is well-known for its role in promoting longevity [274]. In a pilot study we conducted with 8 JSP rats, only 3 rats survived the 10-week study period – a mortality rate of 63%. In this study, JSP rats gained less weight and were often severely debilitated or moribund at time of sacrifice. However, all JSP BB rats survived the study period, had more regular weight gain, and did not demonstrate signs of illness at study end. It is possible that the up regulation of SIRT1 seen in these animals is responsible for some of these benefits. Although this study was not intended to examine the detailed signaling pathways involved in the renoprotective effects of blueberries, but rather to examine the physiological effects of blueberry

supplementation, our findings do present the possibility that one or more novel signaling mechanism is at work. Additional studies are required to confirm these possibilities.

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CHAPTER 6

CONCLUDING REMARKS

Overall Summary of Findings

Hypertension-induced renal disease remains a leading cause of morbidity and mortality in hypertensive patients [275-277]. The redox-responsive transcription factor NF-κB has been shown to contribute to the deleterious effects of reactive species and inflammatory molecules on kidney structure and function [278]. Although obvious putative roles for oxidative stress and NF-κB exist in the development and progression of hypertension-induced renal disease [278-281], the exact signaling mechanisms that perpetuate their effects on the hypertensive kidney remain unclear. The overall hypothesis of this dissertation was that pharmacological or non-pharmacological approaches to reducing both inflammation and oxidative stress would prevent or delay hypertension-induced renal injury in the spontaneously hypertensive rat (SHR), a model for human essential hypertension.

In Chapter 2, we examined the effects of chronic pharmacological NF-κB blockade with pyrrolidine dithiocarbamate (PDTC) on renal dysfunction and mitochondrial redox status in spontaneously hypertensive rats (SHR). PDTC or vehicle was administered orally to 8-week-old SHR and their respective controls for 15 weeks. We showed that PDTC treatment partially attenuated the increase in SBP and normalized renal hemodynamic and excretory parameters and ATP production rates in SHR. PDTC treatment also attenuated the higher levels of cytosolic and mitochondrial ROS generation and tissue mRNA and protein expression levels of NF-κB and oxidative stress genes in SHR without any comparable responses in control rats. These findings suggest that NF-κB activation by ROS induces the cytosolic and mitochondrial oxidative stress

and tissue injury that contribute to renal dysfunction observed in SHR, and highlight a role for NF-κB as a contributor to hypertensive renal disease in this rat model.

In Chapter 3, we focused on exercise as a non-pharmacological method to attenuate NF-κB and decrease oxidative stress in SHR. Normotensive WKY rats and SHR underwent moderate-intensity exercise training, beginning at age 7 weeks, for a total of 16 weeks. Exercise training delayed hypertension, prevented oxidative stress and decreased NF-κB activation in the kidney, while preserving renal hemodynamics and structure in SHR. These results again highlight a role for NF-κB in the oxidative stress and renal dysfunction observed in SHR, and suggest that exercise is a viable non-pharmacological option to decrease renal injury in the SHR model of hypertension.

In Chapter 4, another non-pharmacological approach to preventing hypertension-induced renal disease in the stroke-prone SHR model (SHRSP; a model of more severe renal disease than is seen in SHR) was examined. In this model, we assessed the renoprotective effects of a blueberry (BB)-enriched diet in the setting of hypertension. Blueberries have among the highest antioxidant capacities of fruits and vegetables tested to date and are readily available to consumers, and have been shown to decrease NF-κB activation and ROS production in vivo. Male SHRSP were fed a BB-enriched diet (2% by weight) or an isocaloric control diet for 2 days, 6 weeks, or 12 weeks. In the 6- and 12-week studies, significant decreases in total ROS, peroxynitrite, and superoxide production rates were observed in kidney tissues, which were consistent with reduced pathology and improved function. Additionally, at both time-points, measures of antioxidant status were enhanced. Similar measurements were made in rats fed the same diet for only 2 days, which yielded evidence of increased oxidative stress. This was evidence of hormesis during this short-term exposure, which dissipated with time as indicated by

the initiation of enhanced levels of catalase in the left ventricle and liver of BB-fed rats. Thus, the addition of BB to the diet may be another viable non-pharmacological approach to prevent hypertension-induced renal injury, and may operate via a hormetic effect.

As discussed in Chapter 4, BB-enriched diets are renoprotective and have antioxidant properties in the SHRSP rat. In Chapter 5, we examined the effects of BB in this model in more detail. When fed a 'stroke-permissive' diet (low in protein and altered sodium/potassium ratio) and allowed to drink salt water, mortality will reach 100% in these animals by 15 weeks of age. We examined the effects of BB supplementation of a stroke-permissive diet on mortality, renal function and structure, NF-κB activation, and oxidative stress. SHRSP were fed a stroke-permissive diet or a BB-supplemented stroke-permissive diet from 3 weeks of age to 15 weeks of age. Renal hemodynamic parameters were improved, blood pressure was decreased, ROS production was decreased, and NF-κB activation was decreased in animals fed a BB-supplemented stroke-permissive diet. We also demonstrated that renal pathology was improved in these animals. Further, we found an up regulation of the histone deacetylase, SIRT1, which may be partly responsible for the decrease in NF-κB activation and improved survival seen with BB diet. This study solidifies the possibility that addition of BB to the diet may represent a non-pharmacological option for preventing hypertension-induced renal injury.

Significance of Research

Despite advances in its treatment, hypertension remains the second leading cause of renal failure in the United States [277]. Oxidative stress and inflammation are two major contributors to the progression of hypertension and its associated renal effects [278-281]. Many current antihypertensive therapies modulate both oxidative stress and inflammation in the kidney, but the complexities of hypertension and its renal effects, along with the continued incidence of renal

failure secondary to hypertension, continue to warrant the need for new strategies for and approaches to its treatment.

An abundance of evidence exists that implicates the redox-sensitive transcription factor NF-κB in the progression of oxidative stress and inflammation in the hypertensive kidney [278, 282-288]; however, several mechanisms are believed to contribute to NF-κB activation in hypertension, and these mechanisms are far from completely understood. Thus, a deeper understanding of the interaction between oxidative stress and inflammation in the kidney, and a better understanding of the contributory role of NF-κB to hypertension-induced renal disease is critical to the development of new approaches to prevent or delay its progression.

We believe that our studies have helped identify new pharmacological and non-pharmacological approaches to prevent hypertension-induced renal injury. It is clear that attenuation of both oxidative stress and inflammation in the kidney are crucial to the prevention of hypertension-induced renal damage, and we have shown that targeting NF-κB by drug administration, aerobic exercise, or diet modification is an effective method of mitigating hypertension and preserving renal structure and function. Overall, these proof-of-concept studies have provided a basis for further studies that will advance the understanding of the relationship among NF-κB, oxidative stress, and inflammation in the hypertensive kidney.

Future Directions

Although the aforementioned studies have made significant contributions to identifying a molecular role for NF-κB in perpetuating oxidative stress and inflammation in the hypertensive kidney, further studies are required to understand the underlying molecular mechanisms of NF-κB activation in the kidney. Recent evidence suggests that several other transcription factors can undergo 'cross-talk' with NF-κB, and therefore, the influence of oxidative stress and

inflammation on these transcription factors in the setting of hypertension should be examined. Also, there are several signaling pathways that activate NF-κB and promote oxidative stress; however, it remains unclear which of these pathways is the major contributor to the hypertensive changes in the kidney, and further studies are needed to determine this. NF-κB regulates the transcription of many genes, and the effects of renal NF-κB activation on these genes should also be examined in more detail.

Finally, an understanding of the temporal sequence of events leading to NF-κB activation, oxidative stress, and inflammation in the hypertensive kidney will help us better define appropriate interventions to prevent these deleterious renal effects in the setting of hypertension. In summary, our studies have laid a foundation for a collection of future studies aimed at better understanding the relationship between NF-κB, oxidative stress, and inflammation, and their roles in the development and progression of hypertensive renal injury.

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APPENDIX

LETTER OF PERMISSION

The American Physiological Society

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January 29, 2010

Carrie M. Elks
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Dear Ms. Elks:

The American Physiological Society grants you permission to use the following *American Journal of Physiology Renal Physiology* article in your PhD dissertation for Louisiana State University School of Veterinary Medicine:

Carrie M. Elks, Nithya Mariappan, Masudul Haque, Anuradha Guggilam, Dewan S. A. Majid, and Joseph Francis

Chronic NF-κB blockade reduces cytosolic and mitochondrial oxidative stress and attenuates renal injury and hypertension in SHR
Am J Physiol Renal Physiol 296: F298-F305, 2009. First published December 10, 2008; doi:10.1152/ajprenal.90628.2008

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VITA

Carrie Marie Elks was born to Jeffrey T. and Lynn L. Elks in Baton Rouge, Louisiana. She attended Runnels High School in Baton Rouge and graduated as valedictorian of her class in May of 1996. She attended Louisiana State University on a full scholarship and received her Bachelor of Science degree in human nutrition and dietetics, with Upper Division Honors Distinction, in May of 2001. Carrie immediately relocated to New Orleans, Louisiana, to begin her dietetics internship at Touro Infirmary in August of 2001. Following completion of her internship, Carrie returned to Baton Rouge and passed the Commission on Dietetic Registration national examination to become a registered dietitian in 2002. Carrie practiced for the next four years as a full-time registered dietitian in the fields of cardiology and nephrology and also obtained her Master of Science degree in human nutrition at Louisiana State University in May of 2006. She chose to give up clinical practice to pursue her love for research, and began her studies in the doctoral program in the Department of Comparative Biomedical Sciences at the LSU School of Veterinary Medicine in August of 2006, under the mentorship of Dr. Joseph Francis. Carrie expects to receive her Doctor of Philosophy degree in May of 2011 and will immediately begin a post-doctoral fellowship at Pennington Biomedical Research Center in Baton Rouge.