

2015

Toll-like Receptor 4 (TLR4) in Acute and Chronic Renal Diseases

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TOLL-LIKE RECEPTOR 4 (TLR4) IN ACUTE AND CHRONIC RENAL DISEASES

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program in
Veterinary Medical Sciences through the
Department of Comparative Biomedical Sciences

by

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May 2015

To my grandparents,
who always had aspirations about me but are unfortunately not with me to enjoy this moment

and

To my beloved parents
who have always been my motivational source and a pillar of strength

This dissertation is a testimony of their love

ACKNOWLEDGEMENTS

This journey has been a very enlightening and rewarding experience for me in an area of great personal interest. I would like to acknowledge and express my gratitude to the people who provided generous amount of support and cooperation during this scholarly endeavor. I am deeply indebted to my major professor, Dr. Joseph Francis for having permitted me to work in his lab under his eminent guidance and mentorship. He has been an extremely patient and always encouraging advisor, and made me realize that life is a constant work in progress. I will be forever grateful. I have benefited greatly from the wisdom of my graduate committee members, Dr. Daniel B. Paulsen, Dr. Shaomian Yao, Dr. George Strain and Dr. Bruce F. Jenny. I thank all of them for their time, patience and support. I am grateful to Dr. Inder Sehgal, who was a part of my graduate committee, until he moved to Marshall University in the year 2013. I am extremely thankful to Dr. Venugopal for his timely advices. I am sure; some of his pointers would not only help me in being a good researcher, but will shape how I grow into a better person.

I have gained very much from the guidance, help and friendship provided to me by all members of Francis lab (past and present). I thank Dr. Nithya Mariappan, Dr. Carrie Elks, Dr. Jeffrey Cardinale, Dr. Philip Ebenezer, Dr. Jorge Vila, Dr. Deepmala Agarwal, Dr. Rahul Dange and Dr. Brad Wilson for having contributed in various ways to my work. I also sincerely appreciate Dr. Masudul Haque for teaching me many molecular experimental techniques that I believe would be of great help in my research career. I express my sincere gratitude to all the faculty and staff of the Department of Comparative Biomedical Sciences, for the exemplary support they have provided over the past 5 years. I thank the entire animal husbandry staff in the

Division of Lab Animal Medicine for taking care of the animals I have used in these research studies.

I appreciate the companionship given to me by my friends in the US, without whom I would not have had such a memorable graduate school life. I would like to thank all of them for their encouragement and many good conversations, whether helping each other to solve our problems or just offering emotional support.

Finally, I would like to thank my family for their constant encouragement throughout this undertaking. I have received incredible support from my sister, Asha and all other family members. I owe a special thanks to my fiancée, Akanksha. This dissertation would not have been possible without the succor offered by these individuals.

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LIST OF ABBREVIATIONS

ACE	Angiotensin-converting enzyme
AGE	Advanced glycation end products
AKI	Acute Kidney Injury
Ang II	Angiotensin II
AT1R	Angiotensin 1 receptor
ANOVA	analysis of variance
BB	blueberries
BCA	bicinchoninic acid
BSA	Bovine Serum Albumin
cDNA	complementary DNA
CKD	Chronic Kidney Disease
CMH	1-Hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl pyrrolidine
CPH	1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine
CXCL12	C-X-C motif chemokine 12
CXCR4	C-X-C chemokine receptor type 4
DAMP	danger-associated molecular pattern
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EPR	electron paramagnetic resonance
ESRD	End Stage Renal Disease
FBS	Fetal Bovine serum

GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFR	glomerular filtration rate
HMGB1	High mobility group box protein 1
IACUC	Institutional Animal Care and Use Committee
IL	interleukin
IR	ischemia-reperfusion
LZR	lean zucker rat
MDA	malondialdehyde
MCP	monocyte chemoattractant protein
MetS	metabolic syndrome
mRNA	messenger RNA
NF- κ B	nuclear factor- <i>kappa</i> B
NIH	National Institutes of Health
NLRP3	NOD-like receptor protein 3
O ₂ ^{•-}	superoxide
OONO ⁻	peroxynitrite
OZR	obese zucker rat
PCR	polymerase chain reaction
PEG-SOD	polyethylene glycol superoxide dismutase
PIC	pro-inflammatory cytokines
PRR	pattern-recognition receptors
RAGE	receptor for advanced glycation end-products
RAS	Renin-angiotensin system

RBF	renal blood flow
RNA	ribonucleic acid
ROS	reactive oxygen species
RPF	renal plasma flow
RT-PCR	reverse-transcriptase PCR
RVR	renal vascular resistance
SEM	standard error of the mean
SOD	Superoxide dismutase
TGF	transforming growth factor
TLR	toll-like receptor
TLR4siRNA	TLR4 small interfering RNA
TNF	Tumor necrosis factor
WB	western blot

ABSTRACT

Despite advances in its treatment, the incidence of renal diseases has been consistently increasing. Hence, there is a need to understand the underlying molecular mechanisms of the progression of kidney diseases. Recent research implicates inflammation as an important mediator of renal injury. We hypothesized that inhibiting Toll-like receptor 4 (TLR4), an upstream modulator of several inflammatory pathways, would prevent the progression of renal diseases.

First, we determined the mechanism by which AngiotensinII (AngII)-induced inflammation is modulated by TLR4 using an *in vitro* model of rat tubulo-epithelial cells. We blocked TLR4 using gene silencing strategy in NRK52E cells. In TLR4-silenced cells, the expression of TLR4 was decreased, activation of NF- κ B was reduced, inflammation and oxidative stress were attenuated, suggesting a role for TLR4 in potentiating AngII-induced renal inflammation.

We then focused on an *in vivo* acute kidney injury (AKI) model to elucidate the effect of TLR4 in AKI. We used lipopolysaccharide (LPS), a specific ligand of TLR4, to induce AKI. We injected one group of rats with VIPER, a TLR4 inhibitory peptide, before LPS administration. We also used blueberry as a non-pharmacological approach to study if blueberry could protect against LPS-induced AKI. Compared to LPS-administered rats, the BB-pretreated animals exhibited improved renal hemodynamics, attenuated expression of TLR4 and inflammation. The results in the BB-pretreated group were consistent with the VIPER-treated rats. This indicates that TLR4 is an important mediator in LPS-induced AKI, and suggest that BB, by inhibiting TLR4, is a viable non-pharmacological option to decrease AKI.

We also examined the effect of TLR4 signaling and its downstream mechanism in an animal model of metabolic syndrome-associated chronic kidney disease (CKD) and investigated if a blueberry-enriched diet could attenuate the progression of CKD. We showed that OZR exhibited lower glucose tolerance, exacerbated renal dysfunction and increased oxidative stress. Expression levels of TLR4 and, phosphorylation of ERK and p38MAPK were higher. This was accompanied by increased renal pathology. BB-fed OZR showed significant improvements in all of these parameters. This suggests that the TLR4-MAPK signaling pathway is a key to the renal dysfunction in MetS, and BB protects against this damage by inhibiting TLR4.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 RENAL DISEASES

1.1.1 Introduction

Renal disease research has been of particular interest in the field of medicine over the past decade. Statistics show that the incidence of renal diseases is on the rise, and more than 20 million (one in every 10) American adults have some level of kidney disease (1). Renal diseases are characterized by renal injury markers, reduced kidney function, and altered kidney structure. A renal disease is any abnormality in kidney size or shape or both, and is associated with a decline in the ability of the kidney to function normally for an extended period of time (2). Most renal diseases comprise one or more pathological conditions such as hypoalbuminemia, hyperkalemia or hypokalemia, an elevated serum creatinine and blood urea nitrogen concentration, deposition of excess matrix leading to tubulointerstitial fibrosis (3) and changes in the amount of urine produced. Specific markers of renal dysfunctions need to be identified and further confirmation is to be obtained through diagnostic techniques in order to attenuate the progression of renal diseases. Previous studies have identified urinary biomarkers for progressive renal diseases, with hematopoietic growth factor inducible neurokinin-1 (HGFIN) being one particular biomarker that is increased during pathological conditions of the kidneys (4). In general, if the renal function abnormality continues for more than 3 months, it is considered as end stage renal disease (ESRD)

1.2 ESRD: ADVANCED STAGE (5) OF CHRONIC KIDNEY DISEASE

ESRD is a major cause of mortality as it is the final stage of chronic renal failure. Its occurrence differs depending upon age, sex and race. The Kidney Disease Outcomes Quality

Initiative (KDOQI) has classified chronic renal failure into various stages. Stage 1 consists of kidney damage accompanied by a normal or increased glomerular filtration rate (GFR). A slight reduction in GFR from normal ($> 90\text{ mL/min/1.73m}^2$) i.e $60\text{-}89\text{ mL/min/1.73m}^2$ indicates abnormal blood and urine processing capacity of the kidneys and is considered as Stage 2. In Stage 3, the GFR further reduces to $30\text{-}59\text{ mL/min/1.73m}^2$. Stage 4 has a much more decreased GFR of $15\text{-}29\text{ mL/min/1.73m}^2$ and the severity of the disease at this stage might require a renal transplant. A GFR below 15 mL/min/1.73m^2 is categorized as Stage 5 and this stage necessitates renal replacement therapy (5) or a continuous dialysis process.

1.2.1 Major Causes of ESRD

End-Stage Renal Disease is a highly progressive and irreversible condition. The causes of ESRD include diabetes mellitus, hypertension, chronic glomerulonephritis (6), polycystic kidney disease, obesity (7), oxidative stress, and abnormalities in the renin-angiotensin system. Each of these results in chronic damage to the kidneys via different mechanisms.

Hypertension is a major cause of ESRD (8-11). Kidneys and the vascular system highly depend on each other for good health. The renal system comprises extensive vascular and capillary networks, which supply blood for the proper functioning of the kidneys. A prolonged high blood pressure condition can cause damage to the arterial and capillary systems, causing a reduction in the supply of blood to the kidneys. This induces nephrotic injury, which further affects the ability of the kidneys to properly filter blood and regulate blood pressure, thus contributing to the progression of ESRD (12, 13). Renal failure brings about an increase in the serum creatinine level. Studies have shown that individuals are 3 times more at risk for kidney disease if the serum creatinine level is above 1.7 mg/dL (14). Renin-angiotensin system mediated development of ESRD can also be associated with hypertensive state in patients. Hypertension is

known to induce oxidative stress and inflammation, thus contributing to the progression of kidney diseases.

Oxidative stress and related inflammation cause kidney damage via altering the redox-sensitive pathways. Previous studies have reported an aberrant antioxidant mechanism in ESRD patients (15, 16). The alterations that occur in these antioxidant pathways mainly result in an increased production of reactive oxygen species but the kidneys are either unable to clear these excess oxidants or the antioxidant defense mechanism is impaired (7). The superoxide dismutase activity of the red blood cells has been reported to be reduced in ESRD (17).

Metabolic syndrome has been yet another prominent cause of ESRD in people. Almost half of all ESRD patients are diabetic (18, 19). High levels of blood sugar causes damage to the micro and macrovasculature system within the kidneys and eventually results in proteinuria (20). Obesity and the dyslipidemia associated with obesity have been proven to be major factors that can cause chronic kidney diseases (18). A reduction in the levels of high density lipoprotein and serum apolipoprotein A-1 has been reported in ESRD subjects. Further, an increased low density lipoprotein level and hypercholesterolemia have been characterized in patients affected by ESRD (18). Very low density lipoprotein concentration is also found to be increased in later stages of ESRD and studies have suggested a possible reason for this to be the inability of the kidneys to adequately clear these molecules (21, 22).

1.2.2 Effects of ESRD

Patients with chronic renal failure develop cardiovascular problems that progress along with the severity of the disease. A subject in an early stage of renal failure may also be affected by cardiovascular diseases even though there will only be a small reduction in his/her GFR (23,

24). This can be understood from a deeper review of the major causes of ESRD, most of which are also factors that contribute to cardiovascular diseases. Oxidative stress, inflammation and hypertension, which are major causative agents of ESRD are said to play a pivotal role in the development of cardiovascular problems in these patients. Several studies have shown an increase in the oxidative stress specific biomarkers, such as malondialdehyde and lipid peroxidases, in ESRD patients (25). Furthermore, it has been demonstrated that the concentration of malondialdehyde is inversely proportional to the GFR (17).

Another interesting effect of ESRD is an increased level of adiponectin in the patient's plasma. Usually the adiponectin level is found to decrease in diseased conditions but in the case of chronic kidney diseases, adiponectin levels are found to be higher than normal (26). Researchers are of the opinion that this increased production of adiponectin is to compensate for the metabolic abnormalities that are caused due to ESRD (27).

Proteinuria is another significant effect of chronic renal failure. Proteinuria also has a vicious cycle effect by which it promotes the progression of a chronic kidney disease to ESRD. Due to the decrease in the number of nephrons in chronic kidney disease, the load on the remaining functional nephrons increases in order to maintain the GFR. This excess burden on the nephrons causes damage of the glomeruli in these nephrons. Proteins escape the glomerular filtration membrane and are reabsorbed by the epithelial cells of tubules. These reabsorbed proteins cause the release of many proinflammatory molecules, which further enhance inflammation (28).

Chronic renal disease, as it progresses, leads to a decrease in the hydroxylase activity in the kidneys. This brings about a decline in the serum concentration of 1, 25-dihydroxyvitamin D.

This results in hyperparathyroidism in patients, which is yet another major effect of ESRD (29). Thus ESRD has a wide spectrum of effects on the patient and it is thought that there still exist several causes and effects of ESRD, which are yet to be unraveled.

1.2.3 Current Treatments for ESRD

Since kidneys regulate fluid homeostasis, their proper functioning is critical. Any hindrance to the physiological role of the kidneys can affect the entire body's health. Most major causes of ESRD, like hypertension or diabetes, can be prevented. Angiotensin receptor blockers and inhibitors of angiotensin converting enzyme are used to prevent hypertension-associated renal dysfunctions. Careful monitoring and control of protein intake also aids in the prevention of the progression of chronic renal failure (30). As ESRD is an irreversible pathological condition, the currently available treatments include intermittent dialysis or a renal transplant. Both these treatment techniques are highly expensive and have adverse consequences.

Dialysis is a technique by which the filtration of substances, like water, excess mineral salts and other waste products, by the kidneys is carried out *ex vivo*. Dialysis is initiated only at Stage 4 or Stage 5 of chronic kidney disease. There are different types of dialysis – hemodialysis and peritoneal dialysis being the major ones. Dialysis employs an artificial kidney and a dialyzer. A flow of blood passes from the patient into an artificial kidney, which removes the unwanted substances into the dialyzing fluid via diffusion through a membrane (31).

Renal transplant is yet another current therapy for ESRD. But these techniques have to be strictly monitored to control transplant rejections or adverse immune responses. Rejections, if chronic, are problematic and current treatments are ineffective. Acute humoral rejections are also common in patients (32).

1.3 EXPERIMENTAL MODELS OF RENAL DISEASES

1.3.1 Hypertensive Kidney Disease

The physiological role of kidneys in the development and progression of hypertension is well documented. Maintenance of salt and water homeostasis being the primary function of the kidneys, any change in fluid volume or salt concentration is expected to affect kidney function. Similarly, an elevation in arterial pressure will result in a functional abnormality in the renal vasculature (33, 34), thus contributing to the development of renal diseases.

The animal models of hypertensive renal disease that are widely used include genetic models such as the spontaneously hypertensive rats (SHR) (35) and Dahl salt-sensitive (DS) rats (36). Apart from these genetic models, an angiotensin II treated animal model of hypertension is also used in order to study hypertension-associated kidney disorders (37). The renin-angiotensin system is a major factor in hypertensive renal dysfunction and studies have demonstrated that injecting angiotensin peptide or renin induced hypertension in rats (37). Further it has also been shown that treatment with antagonists or blockers of angiotensin was able to prevent the rise in blood pressure and protect against renal injury in hypertensive rats (37).

1.3.2 Sepsis-induced Acute Kidney Injury (AKI)

AKI is one of the most prevalent forms of kidney diseases that is reported to predispose a person to more complicated kidney disorders such as CKD (38). Sepsis is the most common cause of AKI (39, 40). Sepsis is a multiple organ dysfunction that occurs when an invasive pathogen enters a host. Previous studies proposed that the hemodynamic imbalance and hypotension induced by sepsis results in under-perfusion of the kidneys and this leads to kidney injury (41).

Lipopolysaccharide (LPS) is a component of the gram-negative bacterial cell wall that is used to mimic sepsis in experimental animal models, especially in rodents. LPS injection has been shown to decrease renal blood flow and glomerular filtration rate (42), even without a change in blood pressure (41). LPS also induces the production of proinflammatory cytokines such as TNF α (43), which can further exacerbate the renal injury.

1.3.3 Metabolic Syndrome (MetS) Associated Kidney Disease

MetS was first described in the year 1988 as a combination of selected risk factors that predisposes an individual to cardiovascular and renal diseases (44, 45). The risk factors that comprise the MetS include obesity, hypertension, insulin resistance, dyslipidemia and hyperglycemia (46).

The genetically obese Zucker rat (OZR) is one of the best rodent models of MetS as it expresses all the risk factors involved in MetS. It is also known to closely share the characteristics of obesity in humans (47). These OZR have a mutated leptin receptor and hence are hyperphagic (48). Previous studies have shown an age-dependent reduction in renal function in the OZR compared to their lean controls (LZR). Pamidimukkala J et al and Slyvka Y et al have shown that OZR exhibited reduced renal function parameters, namely GFR and RBF even at 12-14 weeks of age (49, 50).

1.4 OXIDATIVE STRESS IN RENAL DISEASES

A physiological decline in renal function with age has been reported in adults starting from age 30 although this reduction in the efficiency of kidney cannot yet be termed CKD (51). CKD is identified in those who have other risk factors associated with an increased oxidative stress and inflammation (51). Oxidative stress is an imbalance between the rate of production of

free radicals and their degradation by antioxidant enzyme systems that results in an unwarranted accumulation of the radicals (52). The majority of the reactive oxygen species (ROS) generation takes place in the mitochondria during the electron transport chain when some electrons leak from the proper chain and reduce the oxygen molecules to form superoxide radicals (53). The release of these ROS triggers further ROS production via radical chain reactions. During normal physiological conditions, ROS such as hydrogen peroxide, hydroxyl radical, and hypochlorite ion are available at low concentrations where these free radicals play vital roles of being regulators of intracellular signaling mechanisms, nitric oxide synthesis, immune responses and apoptosis (52). At high concentrations, ROS alter various biomolecular functions, and induce pathological processes and result in cellular damage (52, 54). Furthermore, ROS contributes to the synthesis of cytotoxic reactive nitrogen species (RNS) such as peroxynitrite, which can modulate cell signaling and has vasoconstrictive properties (55). Although the short half-life of free radicals makes their detection difficult, there are strong markers of oxidative stress associated with various diseases that have a longer half-life. These markers of oxidative stress that are potential contributors of renal disease pathogenesis include LDL, reactive carbonyl compounds, advanced glycation end-products (AGE) and thiol compounds (52, 56, 57).

Several research and clinical studies have implicated oxidative stress in renal dysfunction (58-60). High malondialdehyde (MDA) levels are considered as an important indicator of cardiorenal abnormalities (61) and accumulation of AGE is associated with chronic renal failure (58). The accumulation of these compounds ensues as a result of the inefficiency of the kidneys to clear them in a timely manner. Kao et al (62) and Droge et al (61) have reported that oxidative stress appears even in the early stages of renal decline. Different mechanisms have been reported for an increased oxidative stress in renal diseases. The prognosis of renal diseases is usually

associated with hypertension, diabetes, low antioxidant enzymatic levels and the inability to clear ROS, all of which can result in an increased oxidative stress in these patients. Further, CKD is a chronic low grade inflammatory disease (63) and this chronic inflammation can itself lead to an increased oxidative stress and contribute to the progression of renal diseases. One of the important inflammatory molecules that has been of particular interest in renal disease recently is the Toll-like receptor 4, which is upstream to several inflammatory cascades.

1.5 TOLL-LIKE RECEPTOR 4 (TLR4) IN RENAL DISEASES

The TLR family of proteins was first identified as “Toll” in *Drosophila* where it was found to play an important role in the innate immune system of the organism (64). Subsequently it was discovered that these TLR proteins were evolutionarily conserved and mammalian TLRs were found to be homologous to the TLR in *drosophila*. TLRs are transmembrane receptors belonging to the IL-1R receptor superfamily (65). Eleven TLR proteins are characterized in humans to date (65). Among these, TLR4 is one of the most studied TLR proteins.

1.5.1 TLR4 and its ligands

TLR4 is critical for the signaling of a variety of ligands. TLR4 is known to be an important component of LPS-induced endotoxic shock (66). The binding of LPS to TLR4 on the cell membrane initiates a sequence of inflammatory reactions resulting in the release of proinflammatory cytokines from immune and non-immune cells. Research studies carried out by different groups have established the importance of TLR4 in LPS signaling. Previous studies found an impaired responsiveness to LPS in mice that had a mutated TLR4 gene (67, 68). TLR4-mutated human patients also showed a hypo-responsiveness to LPS (69). Medvedev et al., showed that phosphorylation of specific tyrosine residues in TLR4 is essential for LPS-induced endotoxin signaling (68).

In addition to LPS, TLR4 can bind a variety of other ligands. The heat shock proteins, Hsp60 (70) and Hsp70 (71) have been shown to bind with TLR4 and activate mitogen-activated protein kinases (MAPK) and NF κ B signaling. Recently, it has been found that TLR4 also acts as a receptor for pathogen associated molecular patterns (PAMP) and danger associated molecular patterns (DAMP), which are mostly endogenous (72-74). An endogenous DAMP that acts as a ligand for TLR4 and initiates an immune response in non-infectious inflammation is HMGB1 (75).

1.5.2 TLR4 signaling in renal diseases

The basic mechanism of ligand binding with TLR4 in infectious and non-infectious cellular injury may be associated with the progression of renal diseases. Emerging evidence indicates that TLR4 plays a crucial role in the pathogenesis of several kidney diseases. However, the exact molecular mechanism by which TLR4 mediates this effect has not been completely elucidated to date. LPS-exposed, murine, renal tubular epithelial cells have an upregulated TLR4 expression, which suggests that TLR4 expression in these cells is associated with sepsis-induced tubular injury (76). Cunningham et al., demonstrated that TLR4-deficient mice were resistant to inflammatory acute renal injury induced by LPS (77). Infectious diseases of the kidney are also predicted to be associated with TLR4 signaling, wherein the renal cells recognize the invading pathogens, activate the macrophages and dendritic cells, and elicit an inflammatory response (78, 79). Even though the major causes of AKI are understood to be either ischemic or nephrotoxic injury, most clinical cases are associated with multiple causes, including sepsis. It is interesting to note that sepsis triggers an activation of TLR4 that can specifically induce downstream NF κ B signaling (76, 80). Therefore, TLR4 could be a possible key to the renal injury and the progression of AKI.

Glomerulonephritis is another renal disorder that has been associated with TLR4. Anti-myeloperoxidase (anti-MPO) is an inducer of glomerulonephritis. It has been found that an increased TLR4 expression was observed in the glomerular cells of mice treated with anti-MPO (81, 82). This injury was concomitant with an increased neutrophil recruitment in the kidneys of these mice. TLR4-deficient mice had an attenuated glomerulonephritis (81). Kidney biopsies from human CKD patients also revealed an increased expression of TLR4, which correlated with increased expression of MCP-1 and TGF- β , which are markers of inflammation and fibrosis respectively (83). Recent studies conducted in animal models of diabetic nephropathy show that TLR4 activation is associated with renal podocyte injury and fibrosis in these animals. Again, TLR4 knockout mice exhibited resistance to the development of diabetic nephropathy, which was associated with attenuated inflammation and cellular injury (84). All of these previous findings clearly indicate a vital role for TLR4 in the development of renal diseases but the exact mechanism of its action is not yet completely understood.

1.6 HIGH MOBILITY GROUP BOX 1 (HMGB1) PROTEIN IN RENAL DISEASES

High mobility group box 1 (HMGB1) is an evolutionarily conserved non-histone nuclear binding protein and an endogenous ligand for TLR4 and receptor for advanced glycation end-products (RAGE) (85-87). In cells, HMGB1 is known to have the ability to shuttle between the nucleus and cytoplasm, although in normal conditions its presence is restricted to the nuclei to bind chromatin (88). During tissue injury, HMGB1 is released by activated immune cells and/or injured cells into the extracellular milieu. Extracellular HMGB1 functions as a danger-associated molecular pattern (DAMP) which can induce robust activation of the innate immune system (89) and also acts as a major proinflammatory mediator of inflammatory diseases (86). HMGB1 has been reported to contribute to the progression of several chronic inflammatory diseases including

atherosclerosis (90). Research evidence suggests that HMGB1 plays an important role in renal diseases, such as acute kidney injury (91, 92), glomerulonephritis (93), diabetic nephropathy (94), lupus nephritis (95) and renal allograft rejection (96).

1.6.1 Structure of HMGB1

HMGB1 is the most highly expressed high mobility group protein, with about a million molecules present in a single cell (97). HMGB1 is a 215 residue protein organized into two separate DNA binding domains, namely the N-terminal A-box and the B-box. In addition to these DNA-binding domains, the structure of HMGB1 also contains an acidic tail at the C-terminal end. The A-box and the B-box each are formed of approximately 75-80 residues and the C-tail has about 30 amino acids (96). The B-box comprises the binding sites for TLR4 (residues 89-108), the major ligand of HMGB1 that is responsible for its proinflammatory and cytokine activity (98, 99) whereas the A-box acts as an antagonist of the endogenous HMGB1 protein (99), thereby serving to inhibit the proinflammatory effects of cytoplasmic HMGB1 (100).

1.6.2 HMGB1 release and signaling

Intranuclear HMGB1 is a DNA binding protein that regulates a number of DNA-associated activities including nucleosome stability, nucleosome release, gene transcription, genome chromatinization, DNA replication and DNA repair (97). In addition to these intracellular roles, HMGB1 has been identified to play a vital role as an extracellular mediator of inflammation (92, 96). The translocation of HMGB1 from the nucleus to the extracellular environment is described to occur in two different ways. Activated immune cells such as macrophages, monocytes and dendritic cells secrete HMGB1 (active pathway) in response to inflammation (101, 102) and damaged or dying cells release HMGB1 in a passive manner (96). Immune cells upon activation by LPS undergo acetylation of lysine residues, which is a signal

for HMGB1 translocation (102). Although the exact mechanism of HMGB1 release is yet to be elucidated, research findings suggest that the inflammasomes, an innate immune system component, play a major role in this process. Lamkanfi et al showed that HMGB1 release from LPS-exposed macrophages required the activation of NLRP3 inflammasome (103).

A growing body of evidence indicates that HMGB1 can bind to multiple receptors, such as TLR2 (104), TLR4 (87, 105, 106) and RAGE (106), in the kidneys during inflammatory injury. Different release mechanisms induce the release of different redox forms of HMGB1. Necrotic cells trigger the release of a fully-reduced isoform of HMGB1, which then forms a heterodimer with the CXCL12 chemokine to signal through CXCR4 receptor. Apoptotic cells release an oxidized HMGB1. The oxidized isoform of HMGB1 is not known to induce cytokine production or chemotaxis. Activation of the TLR4 ligand induces the release of disulfide HMGB1 that has thiol groups on C106 and a disulfide bond between C23 and C45 (102). HMGB1-TLR4 interaction has been demonstrated to signal via the MyD88-dependent pathway (87, 102). The binding of HMGB1 to these receptors result in the activation of NF κ B signaling and subsequent inflammatory cascade by promoting the upregulation of proinflammatory cytokines (96).

The ability of HMGB1 to activate the production of cytokines and chemokines has led to investigation of its role in renal diseases. HMGB1 has been implicated in a variety of kidney diseases, including glomerulonephritis, wherein HMGB1 levels were found to be elevated in the urine and serum of patients. HMGB1 also induced the activation of monocyte chemoattractant protein-1 (MCP-1) in these patients resulting in further translocation of HMGB1 from the nucleus to cytoplasm (107). Wang et al., demonstrated that HMGB1 acts as a mediator of septic inflammation (101), which underlines the role played by HMGB1 in AKI as it is associated with

sepsis-related complications. Injured renal cells in renal ischemia-reperfusion (IR) have been shown to release HMGB1, which binds to TLR4 receptor in leukocytes to induce the production of inflammatory cytokines (108). Further, a time-dependent increase in HMGB1 levels were demonstrated in the kidneys of rats after an IR injury (109). HMGB1 has been studied in diabetic nephropathy and the results show an increase in the expression of HMGB1 in diabetic rat kidneys (110). This also suggests HMGB1 as a possible mediator of diabetic renal injury. In CKD patients, HMGB1 levels were reported to correlate with GFR and the extent of inflammation (111). Further extensive studies are required to elucidate the exact mechanism of the role played by HMGB1 in kidney diseases.

1.7 STATEMENT OF THE PROBLEM AND SPECIFIC AIMS

A chronic inflammatory condition and oxidative stress environment induce a vicious cycle that can contribute renal injury and result in its progression to end stage renal disease. As a major inflammatory molecule that is upstream of several important inflammatory cascades, TLR4 activation has been implicated in the injurious effects of inflammation on the renal structure and function. Although previous studies have proposed a vital role for TLR4 in acute and chronic renal diseases, the exact signaling mechanisms by which TLR4 exerts its inflammatory effect in the kidneys are still poorly understood.

The overall objective of this dissertation was to elucidate the exact signaling mechanisms by which TLR4 contributes to the development of different models of acute and chronic kidney diseases. We hypothesized that pharmacological and non-pharmacological approaches, which can inhibit the expression of TLR4, would prevent or delay the progression of renal diseases in different models of acute and chronic kidney diseases. We performed a series of *in vitro* and *in vivo* experiments, to fulfill the following specific aims:

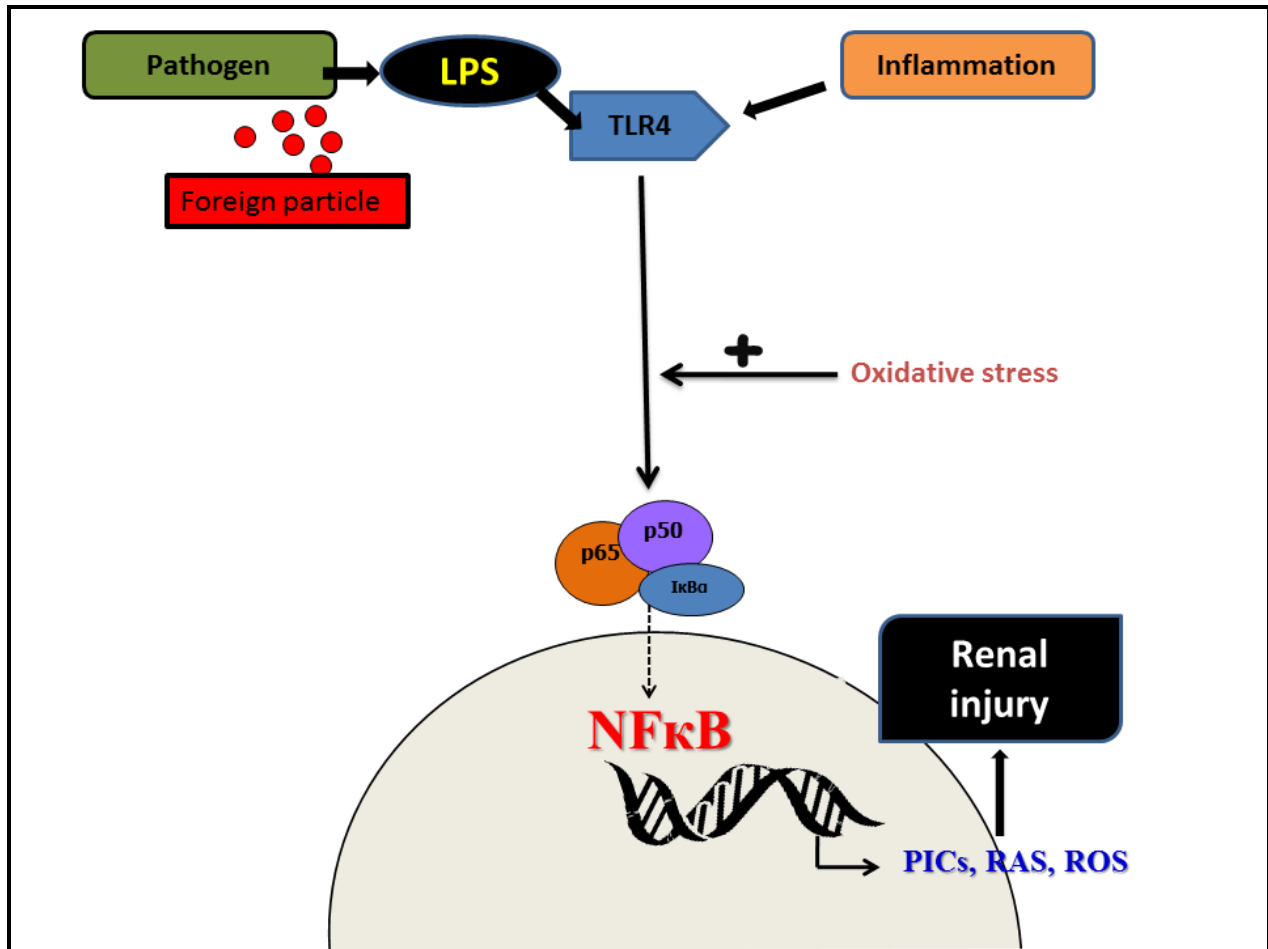


Figure 1.1: The proposed signaling mechanism of the inflammatory activity of TLR4 on the kidney.

Objective 1: Determine how HMGB1-TLR4 signaling mediates the angiotensin II-induced hypertensive renal injury in an *in vitro* model of rat tubular epithelial cells.

Objective 2: Examine the role of TLR4 in a lipopolysaccharide-induced acute kidney injury (AKI) model and determine the beneficial effects of a blueberry supplementation in this animal model.

Objective 3: Investigate the effects of a blueberry-enriched diet in improving renal function and reducing oxidative stress by modulating TLR4 expression in an animal model of metabolic syndrome-associated chronic kidney disease.

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CHAPTER 2

ANGIOTENSIN II-INDUCED HYPERTENSIVE RENAL INJURY IS MEDIATED THROUGH HMGB1-TLR4 SIGNALING IN RAT TUBULO-EPITHELIAL CELLS

2.1 INTRODUCTION

Hypertension is one of the most critical predisposing factor for the development of end-stage renal disease (ESRD). According to the American Heart Association, about one-third of the adult population has high blood pressure (1). While effective control of hypertension has been implemented by medications such as angiotensin-converting enzyme inhibitors and angiotensin-receptor blockers, the clinical course of the disease remains progressive and its prevalence is on the rise (2). Consequently, new and innovative approaches for treating hypertension are needed. Inflammation is an important risk factor for end-organ damage (3-6) and has been implicated in hypertension (7, 8). A growing body of evidence suggests that inflammatory cytokines play important roles in the pathophysiology of renal disease (5, 9). Several groups have implicated the role of circulating cytokines in ESRD (10-12). These proinflammatory cytokines (PIC) cause an over-activation of the renin-angiotensin system (RAS), which also plays a major role in the pathogenesis of ESRD (13).

Our previous reports have shown that blocking inflammatory molecules and their transcription factor, nuclear transcription factor kappa B (NFkB), attenuates sympathetic activity (14, 15) and hypertension (8). However, it remains unknown how inflammation is activated during the development of hypertension. We proposed that the interaction between the inflammatory cytokine protein high-mobility group box 1 (HMGB1) and toll-like receptor 4, TLR4, results in the up-regulation of NFkB, thereby contributing to the development of hypertension.

HMGB1 is a potent innate danger/damage associated signaling molecule, which is actively secreted by immune cells and necrotic cells (16). The release of HMGB1 marks the initiation of host defense or inflammatory tissue repair mechanisms (17). Receptor blocking studies have shown that HMGB1 is a strong endogenous ligand of TLR4 receptor (18-21). TLR4, a receptor found on the cell surface, mediates the proinflammatory effects (22) of its ligands through the NFkB signaling pathway. HMGB1 has been shown to be a pivotal factor in the pathogenesis of inflammatory diseases such as autoimmune disease (23), endotoxin-induced sepsis (24) and ischemia/reperfusion injury (25).

The objective of this study was to understand the molecular mechanisms contributing to Angiotensin II (AngII)-mediated kidney injury. In this *in vitro* study, we show that HMGB1 and its receptor TLR4 are activated in response to AngII insult in the NRK52E rat tubular epithelial cell line. Gene silencing of TLR4 and inhibition of HMGB1 attenuates the AngII-mediated, inflammatory injury in these cells. Our data provide strong evidence for the involvement of HMGB1-TLR4 signaling in the development of hypertensive renal injury.

2.2 MATERIALS AND METHODS

Renal tubular epithelial cell culture – NRK52E cell line

Cell lines were grown in Dulbecco's modified eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 100 IU/ml penicillin, at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Unless otherwise stated, cells were plated at a density of 4 x 10⁶ cells per 60mm dish or 1 x 10⁷ cells per 100mm plate. All experiments were performed at 70-80% confluency. All treatments were done in serum-free medium. To study the effect of AngII on inflammatory cytokines, NRK52E cells were exposed to AngII or vehicle (control). We performed a pilot experiment to validate the best time-point for AngII stimulation of NRK52E cells, using a

previously reported (26, 27) concentration (1 μ M) of AngII in culture medium over a stipulated time course (0-24 h). At defined time-points, cells were harvested for real-time PCR for mRNA analysis of TLR4, HMGB1 and proinflammatory cytokines, such as TNF α and IL-1 β . We observed that 6h was the earliest time-point at which an AngII-exerted effect was evident at the mRNA level. AngII treatment for 24h did not cause any significant changes in the expression of these genes when compared with 6h. In all subsequent experiments, cells were exposed to 1 μ M AngII for 6h. All results are presented as mean \pm SD or mean \pm SEM, and represent set of three independent experiments in NRK52E cells. In each experiment, n=6 per treatment groups were used unless otherwise stated.

Treatment of cells with Losartan

In another set of experiments, cells were pre-treated with Losartan (100 μ M) for 2h before AngII exposure for 6h. The treatment dose for losartan was obtained from previous studies conducted on the same cell line (27). Following exposure to AngII receptor blocker, cells were harvested for real-time PCR, and western blot analysis.

TLR4 siRNA construction and transfection

We explored the effects of TLR4 silencing by using a gene knock-down approach, RNA interference through the delivery of small interfering RNA (siRNA) against TLR4 (Santa Cruz Biotechnology, CA, USA). A scrambled sequence of the same length was used as a control. All treatments were done by following the manufacturer's recommended protocols. Twenty four hours after plating, NRK52E cells were transfected separately in 6-well plates with TLR4siRNA or the scrambled control sequence for 24 hours prior to subsequent treatments.

HMGB1 neutralization

All treatments were performed following the manufacturer's recommended protocol. HMGB1 neutralization was achieved by pretreating the cells for 4 hours using chicken anti-HMGB1 neutralizing polyclonal antibody (IBL International Corp, ON, Canada) at a dosage of 1µg/ml (28).

RNA extraction and Real-Time PCR

Semi-quantitative real-time PCR was used to determine the mRNA levels of TLR4, HMGB1, TNFα and IL-1β in NRK52E cells. Total RNA isolation, cDNA synthesis and RT-PCR were performed as previously described (29, 30). Semi-log amplification curves were evaluated by the comparative quantification method ($2^{-\Delta\Delta Ct}$), and GAPDH or 18s RNA was used for normalization of all reported gene levels. The data presented are the fold changes of the gene of interest relative to that of the control group.

Western blot analysis

For whole cell extracts, cells were washed twice with ice-cold PBS and scraped into 100µl per dish of cell lysis buffer (Cell Signaling Technology Inc, Danvers, MA, USA), containing protease and phosphatase inhibitors. Samples were incubated on ice for 10 min and then centrifuged (12000 rpm, 5 mins, 4°C). The supernatants were retained. Protein concentrations were determined by bicinchoninic acid (BCA) method, lysates were stored at -80°C until used for western blotting.

Cell lysates were mixed with Laemmli sample buffer (Bio-Rad Laboratories, CA, USA) and placed in a boiling water bath for 5 min. Proteins (30µg) were separated by SDS-PAGE using 10-15% resolving gels and 6% stacking gels, and then transferred onto a PVDF membrane. Non-specific binding sites were blocked with 5% (w/v) BSA in Tris buffered saline with tween-

20 (TBST). Blots were probed overnight with primary antibodies. Specific primary antibodies included anti-HMGB1 (1:1000), anti-TLR4 (1:1000) and anti-TNF α (1:1000, Santa Cruz Biotechnology Inc., Texas, USA). Blots were washed with TBST, incubated with HRP-conjugated secondary antibodies (1:10000) for 1 hour. Bands were visualized using enhanced chemiluminescence (ECL Plus, Amersham, NJ, USA). Band intensities were quantified using ImageJ software.

Flow Cytometry

The cultured cells were trypsinized using 0.25% trypsin-EDTA and collected. DMEM was added to arrest the enzymatic action of trypsin and the cells were spun at 2000rpm for 5 mins. The pellets of cells were re-suspended in 1X PBS. To study TLR4 expression, 1×10^6 cells were incubated with anti-TLR4 (1:250) antibody for 30 minutes at room temperature. The samples were washed using 1X PBS to remove any unbound antibody and then incubated with Alexa Fluor 488 goat anti-rabbit IgG antibody (1:250) in 10% normal goat serum-PBS for 30 minutes at room temperature. Samples were again washed. Autofluorescence and secondary antibody controls were also prepared.

Samples were analyzed on a FACScan flow cytometer (BD Biosciences, San Jose, CA) utilizing a 15 mW 488 nm argon-ion laser and configured for Alexa Fluor 488 fluorescence measurements using log amplification. Data from total of 10,000 cells per sample were acquired on a Macintosh G5 workstation (Apple Computer, Cupertino, CA) running Cellquest Pro software (BD Biosciences, San Jose, CA). Cellular artifact was eliminated by gating on intact cells based on dot plots of forward scatter versus side scatter. Fluorescence analyses in the form of histograms were generated using Cellquest Pro software (BD Biosciences, San Jose, CA).

Percentages of cells expressing TLR4 were determined for each sample based on comparisons with negative controls.

Electron paramagnetic resonance spectroscopy (EPR)

Total ROS and superoxide production rates were measured in NRK52E cells via EPR spectroscopy as previously described (29-33). The term ‘total ROS’ represents all ROS; however, the major ROS detected by this method were superoxide, hydrogen peroxide, and hydroxyl radical, with other species as minimal contributors. Briefly, cells were incubated at 37°C with 6.6µl of CMH (200µM) for total ROS measurement; 1.5µl of PEG-SOD (50U/µl) for 30 minutes, then CMH for an additional 30 minutes for superoxide measurement. Aliquots of incubated probe media were taken in 50µl disposable glass capillary tubes (Noxygen Science Transfer and Diagnostics, Elzach, Germany) for determination of total ROS or superoxide production, using previously established EPR settings (29, 32)

HMGB1 ELISA

The cell culture medium was collected in sterile tubes, spun at 2000 RPM for 10 minutes. The supernatant was collected and stored at -80°C. HMGB1 concentrations in the medium was measured using the rat high mobility group protein B1 (HMGB1) ELISA kit (MyBioSource Inc., San Diego, CA) using the manufacturer’s recommended protocol.

Quantification of NFκB p65 activity

The NFκB p65 activity ELISA (Active Motif, USA) kit was used to assess the binding activity of free NFκB p65 in nuclear extracts, as described previously (29). A sandwich ELISA method was employed to perform the analysis, according to the manufacturer’s instructions.

Statistical analysis

Statistical analysis was performed either using paired t-test or one-way ANOVA with Bonferroni post hoc test using Prism software (GraphPad Software, CA, USA). Data are presented as the fold change of each gene of interest relative to controls. Results were considered significant when $p < 0.05$.

2.3 RESULTS

AngII causes inflammatory activation of HMGB1 and TLR4 in NRK52E cells

To investigate the influence of AngII on HMGB1 and TLR4 in renal tubular epithelial cells, we treated NRK52E cells with AngII (1 μ M) for stipulated time-points and examined the mRNA levels of HMGB1 and TLR4 in whole cell extracts. We also determined the gene expression levels of proinflammatory cytokines TNF α and IL-1 β . We observed that AngII treated cells exhibited significant increases in HMGB1, TLR4 and the PIC after 6h of exposure (Figure 2.1).

AngII exposure (6h) resulted in a seven-fold increase in TLR4 and HMGB1 mRNA expression, a ten-fold increase in TNF α mRNA expression and a six-fold increase in IL-1 β mRNA expression. There was no significant change in the expression of these transcripts after 24h AngII treatment in comparison with 6h treatment.

Effects of AngII treatment on TLR4, HMGB1 and PIC expression levels in NRK52E cells

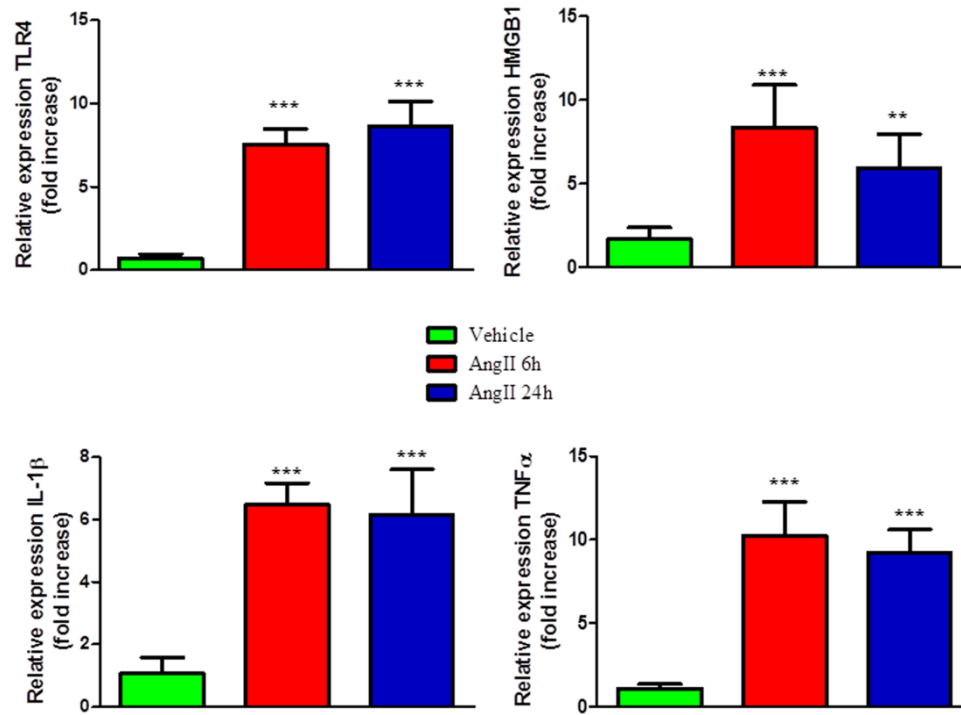


Figure 2.1: Effect of AngII treatment on TLR4, HMGB1 and PIC expression levels in NRK52E cells. Serum starved rat tubulo-epithelial cells were treated with AngII on a time-dependent basis and mRNA expression of TLR4, HMGB1, and the proinflammatory cytokines TNF α and IL-1 β were determined. The results are mean \pm SEM of three independent experiments (n=12 per experimental group, **p < 0.01, ***p < 0.001).

Treatment with losartan decreased the expression of TLR4, HMGB1 and PIC in NRK52E cells

To confirm that the inflammatory activation of HMGB1 and TLR4 was AngII-induced, we pretreated NRK52E cells with losartan (100 μ M), an AT1 receptor blocker. We determined the mRNA expression levels (Figure 2.2A) of HMGB1, TLR4, TNF α and IL-1 β and protein expression (Figure 2.2B-E) levels of HMGB1, TLR4 and TNF α in these cells. The mRNA and protein expression levels of HMGB1, TLR4 and TNF α were elevated in AngII treated cells. Losartan pretreatment for 2 hours significantly reduced the mRNA and protein expression of HMGB1, TLR4 and TNF α in renal tubular epithelial cells. Additionally, losartan treatment significantly attenuated the increase in mRNA expression of IL-1 β in these cells. As expected, there was no difference in gene or protein expression between Vehicle and Vehicle+Los groups.

Inhibition of TLR4 by RNAi attenuated AngII-mediated production of proinflammatory mediators in NRK52E cells

AngII-treated cells had significantly increased levels of TLR4 and HMGB1 compared with control cells (Figures 2.2 and 2.3). Interestingly pretreatment of cells with TLR4siRNA resulted in a significant reduction in mRNA (Figure 2.3A) and protein expression levels (Figure 2.3B-E) of HMGB1, TLR4 and TNF α . We also observed that the increased expression levels of IL-1 β mRNA was attenuated in cells treated with TLR4siRNA. There were no differences between Vehicle and Vehicle+TLR4siRNA groups. These results demonstrate that pretreatment of cells with RNAi silencing TLR4 causes reversal of AngII-induced proinflammatory mediators in NRK52E cells.

Effects of pre-treatment with Losartan before AngII exposure in NRK52E cells

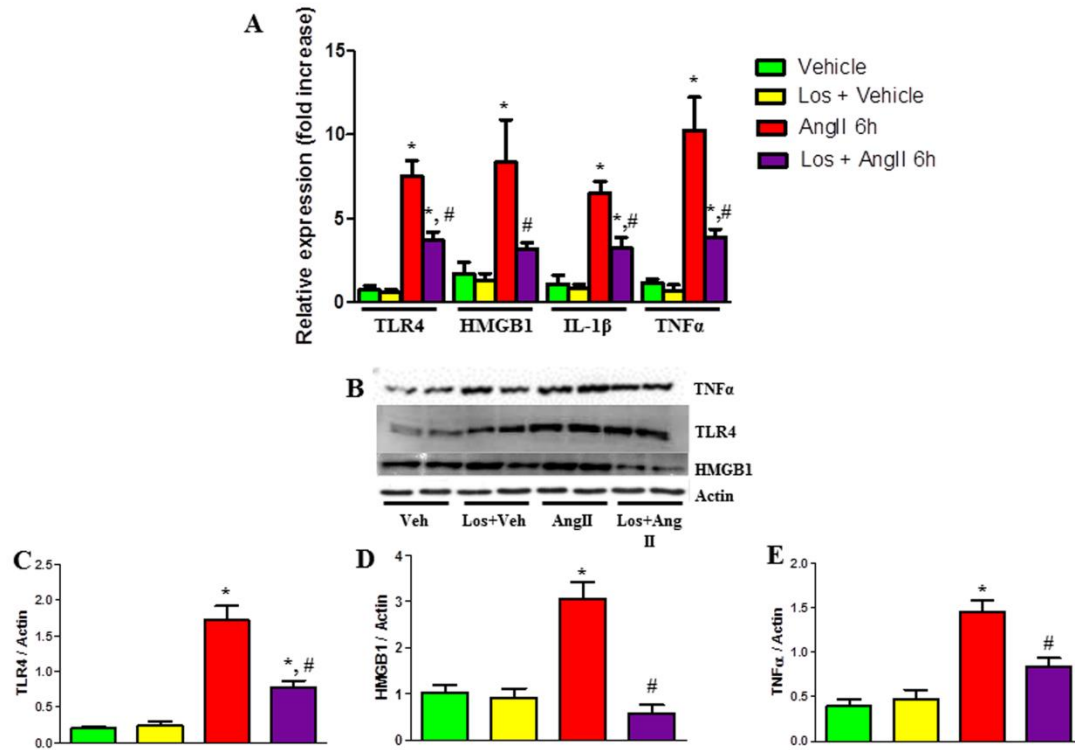


Figure 2.2: Effect of pretreatment with Losartan before AngII exposure in NRK52E cells. Serum starved NRK52E cells were pre-treated with losartan for 2 hours before AngII exposure. (A) mRNA expression levels of TLR4, HMGB1, TNF α and IL-1 β . (B) Representative western blots. Densitometric analysis of western blot results showing protein expression of (C) TLR4, (D) HMGB1 and (E) TNF α . All values are presented as mean \pm SEM (*p < 0.05 vs. Vehicle, #p < 0.05 vs. AngII 6h treated cells)

Inhibitory effects of TLR4siRNA on AngII-mediated inflammation in NRK52E cells

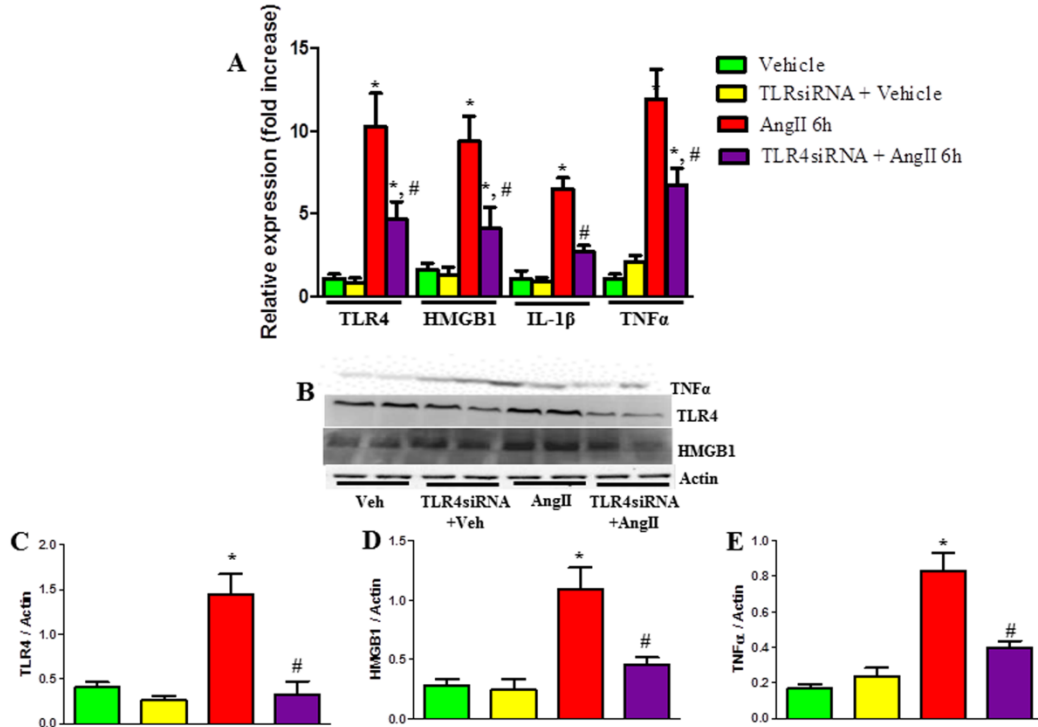


Figure 2.3: Inhibitory effects of TLR4siRNA on AngII-mediated inflammation in NRK52E cells. Serum starved NRK52E cells were transduced with TLR4siRNA for 4 hours before AngII exposure. (A) mRNA expression levels of TLR4, HMGB1, TNFα and IL-1β. (B) Representative western blots. Densitometric analysis of western blot results showing protein expression of (C) TLR4, (D) HMGB1 and (E) TNFα. All values are presented as mean ± SEM (*p < 0.05 vs. Vehicle, #p < 0.05 vs. AngII 6h treated cells)

AngII-mediated production of proinflammatory mediators was attenuated with anti-HMGB1 treatment in NRK52E cells

To demonstrate effects of HMGB1 in AngII-induced production of proinflammatory molecules in renal epithelial cells, NRK52E cells were pretreated with anti-HMGB1 and the expression of HMGB1, TLR4 and PIC in these cells was examined. We observed that there was no difference between Vehicle and Vehicle+antiHMGB1 treatment groups. As determined previously, there was a significant elevation in the expression of HMGB1, TLR4 and PIC in AngII-exposed cells (Figure 2.4A-E). This increase in the expression of inflammatory molecules was reduced with anti-HMGB1 pretreatment in NRK52E cells. These results indicate that AngII-induced activation of the inflammatory cascade is mediated by HMGB1-TLR4 interaction in kidney epithelial cells.

We also measured HMGB1 in the medium to assess the levels of HMGB1 released by the cells upon treatment. There was a significant increase in the circulating (media) HMGB1 levels in AngII treated cells (Figure 2.4F). Anti-HMGB1 treatment or TLR4 silencing in rat tubular epithelial cells inhibited this increase in HMGB1 release. This result indicates that AngII-induced inflammatory injury promotes the release of HMGB1 into the milieu and by treating the cells with anti-HMGB1 or TLR4siRNA, AngII-induced proinflammatory cytokine production and subsequent injury can be significantly decreased.

TLR4 siRNA and anti-HMGB1 treatment decreased the number of TLR4-positive NRK52E cells

We performed flow cytometry on the NRK52E cells to determine if AngII treatment increased the number of TLR4-positive cells and if pretreatment with TLR4 siRNA or anti-

HMGB1 reduced the number of these cells. We observed that AngII treatment induced a significant increase in the number of TLR4-positive NRK52E cells (Figure 2.5).

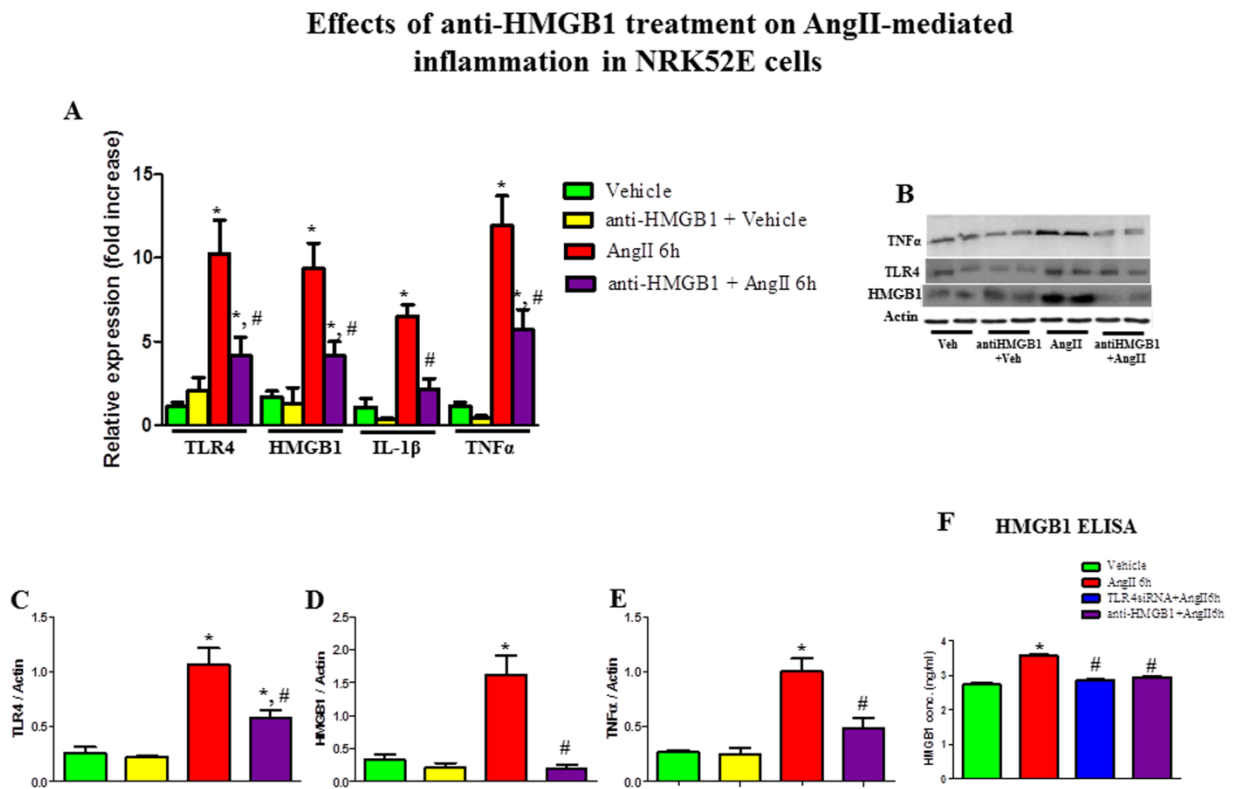


Figure 2.4: Effects of anti-HMGB1 treatment on AngII-mediated inflammation in NRK52E cells. Serum starved NRK52E cells were treated with neutralizing anti-HMGB1 for 4 hours before AngII exposure. (A) mRNA expression levels of TLR4, HMGB1, TNFα and IL-1β. (B) representative western blots and densitometric analysis of western blot results showing protein expression of (C) TLR4, (D) HMGB1 and (E) TNFα. (F) HMGB1 ELISA performed in cellular supernatants obtained after treatment of NRK52E cells to determine the level of HMGB1 release. All values are presented as mean ± SEM (*p < 0.05 vs. Vehicle, #p < 0.05 vs. AngII 6h treated cells)

Consistent with our RT-PCR and western blot results, there was a significant reduction in the number of cells positive for TLR4 upon pretreatment with TLR4 siRNA or anti-HMGB1. These data suggest that the HMGB1 result in the upregulated expression of TLR4 that is attenuated in anti-HMGB1 treated cells.

Gene silencing of TLR4 and anti-HMGB1 treatment attenuated NFκB activation and reduced the rate of generation of reactive oxygen species in NRK52E cells

The activation of TLR4 was confirmed by measuring the NFκB activity. We measured the NFκB p65 DNA binding activity in cells from all treatment groups. Consistent with the TLR4 expression results, cells exposed to AngII had significantly higher NFκB activity compared with the control cells (Figure 2.6A). The TLR4 siRNA transfected cells had a significantly lower NFκB activity levels compared with the AngII treated cells suggesting that gene silencing of TLR4 inhibited the increase in AngII-induced NFκB activity seen in renal epithelial cells. The cells that were pretreated with anti-HMGB1 also exhibited an attenuated NFκB activity.

TLR4-induced inflammation is known to elevate the generation of reactive oxygen species (ROS). We measured the rate of generation of total ROS and superoxide in NRK52E cells from all experimental groups (Figure 2.6B-C). There was no change in the rate of generation of ROS between control cells and control cells treated with either of these blockers. We observed a significant increase in the rate of production of total ROS and superoxide in cells that were exposed to AngII. In contrast, pre-treatment with TLR4 gene-silencer and anti-HMGB1 attenuated this AngII-mediated increase in total ROS and superoxide levels in NRK52E cells.

Effect of TLR4siRNA and anti-HMGB1 treatment on AngII-mediated increase in the number of TLR4-positive NRK52E cells

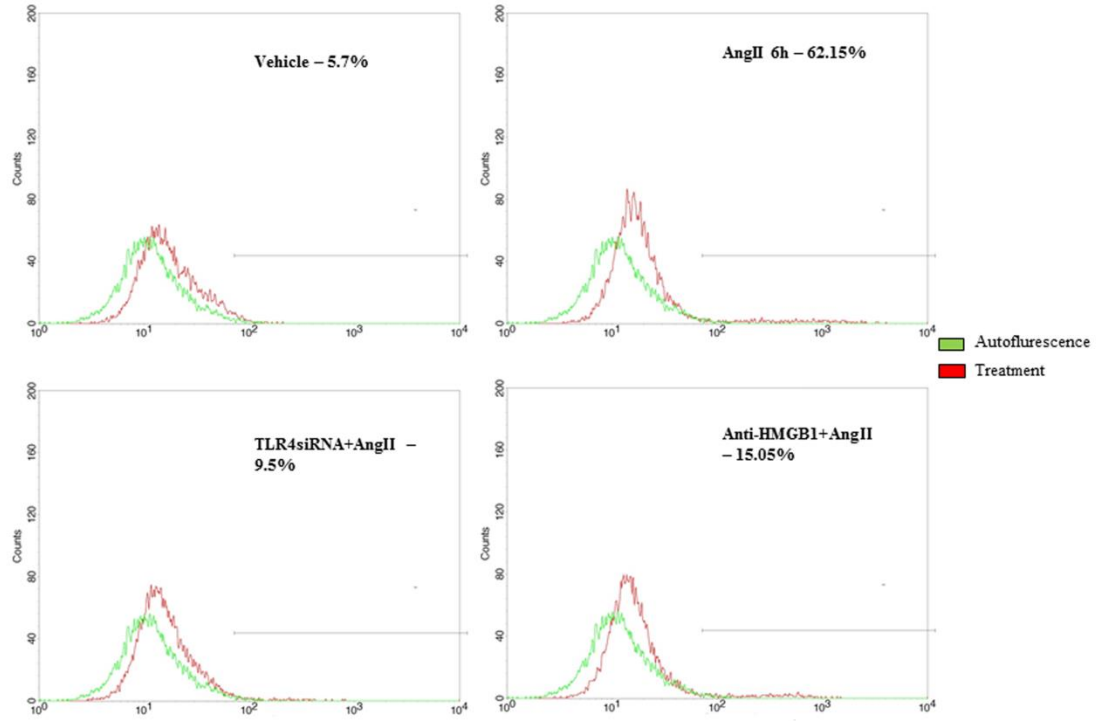


Figure 2.5: Effects of TLR4siRNA and anti-HMGB1 treatment on AngII-mediated increase in the number of TLR-positive NRK52E cells (n=9 per experimental group).

**Inhibitory effect of TLR4siRNA and anti-HMGB1 treatment on
AngII-mediated NFκB activation and rate of generation of ROS in
NRK52E cells**

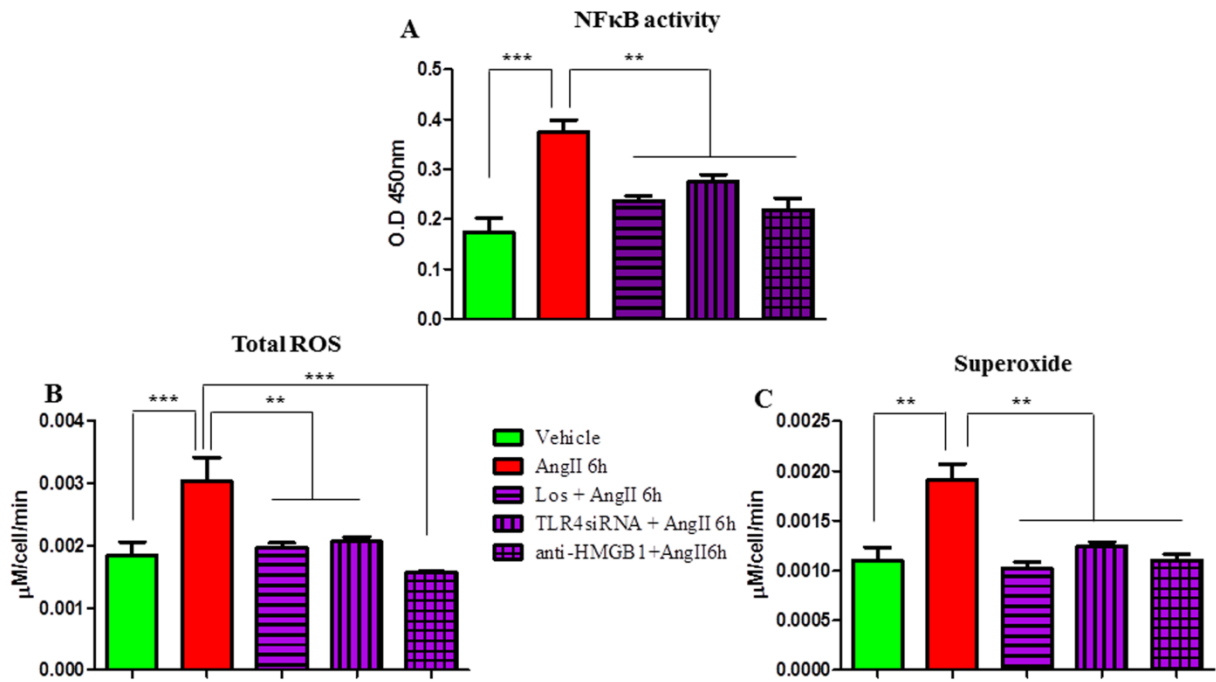


Figure 2.6: Inhibitory effects of TLR4siRNA and anti-HMGB1 treatment on AngII-mediated NFκB activation and rate of generation of ROS in NRK52E cells. (A) DNA binding activity of NFκB p65 subunit in rat renal tubulo-epithelial cells from each experimental group (n=8), as determined by ELISA. Rate of generation of (B) Total ROS and (C) Superoxide in the NRK52E cells. All values are presented as mean \pm SEM (**p < 0.01, ***p < 0.001)

2.4 DISCUSSION

The objective of the present study was to investigate the underlying molecular mechanisms by which AngII induced renal injury occurs, and to elucidate the role of HMGB1-TLR4 signaling in mediating this effect. The salient findings of this study include: 1) AngII causes activation of the TLR4 inflammatory cascade in renal tubular epithelial cells and subsequent up-regulation of PIC; 2) AngII causes an increase in the expression of HMGB1 in NRK52E cells; 3) gene-silencing of TLR4 and blockade of HMGB1 reversed the AngII-induced effects, suggesting an important role for the TLR4-HMGB1 signaling pathway in mediating AngII mediated renal injury. The results of this study support a novel molecular mechanism that AngII-induced, increased secretion of HMGB1 leads to over-activation of TLR4, which then regulates NF κ B to induce the production of PIC. Our data also suggests that AngII-mediated renal injury can be ameliorated by inhibiting HMGB1 and TLR4 separately, indicating that these molecules are potential therapeutic targets in inflammatory renal diseases.

Inflammation is one of the important hallmarks of hypertension and subsequent renal injury. TNF α (34), IL-1 β (35) and other circulating PIC (35, 36) have been reported to increase in the kidney with the severity of hypertension. However, the mechanisms by which hypertension contributes to the progression of renal injury is not clear. In this study, we observed that AngII exposure resulted in significant up-regulation of TNF α and IL-1 β in NRK52E cells after 6hrs. Our results are consistent with those of previous studies which have shown that AngII infusion increases the production of PIC in rat kidneys (37-39). Although previous studies have investigated the effects of AngII on kidneys, the upstream signaling mechanism are not well understood. Therefore, we examined the effect of AngII on TLR4, which is an important upstream receptor molecule, which can trigger an immune response and, on HMGB1, which is

an important inflammatory molecule and an endogenous ligand of TLR4. Our data show that AngII treatment for 6hr caused an increased expression of HMGB1 and TLR4 in the renal tubular epithelial cells.

Angiotensin is a vaso-constrictive peptide known to regulate the blood pressure homeostasis. The activation of the vaso-constrictive arm of the RAS contributes to the progression of many inflammatory diseases. Anti-inflammatory effects of angiotensin receptor blockers (ARB) have been well studied in clinical cases of hypertension and other cardio-renal abnormalities including stroke (40) and sepsis (41). Despite these studies, there have not been many reports that have implicated the involvement of TLR4 signaling on the AngII-induced renal injury model. Hence, we studied the effect of losartan (an ARB) on the inflammatory signaling cascade activated by TLR4. Our results clearly show that blockade of angiotensin receptors attenuate the TLR4-mediated inflammation by down-regulating TLR4 and HMGB1 expression levels. In addition to attenuated TLR4 levels, pretreatment with losartan also reduced the expression levels of the downstream PIC, TNF α and IL-1 β .

Recent research studies have implicated the involvement of toll-like receptors (TLR), which are important mediators of innate immune response, in several inflammatory disease conditions (29, 42-45). TLR are cell surface glycoprotein receptors that recognize pathogens or danger-associated molecular patterns, trigger the innate immune response (46), and are shown to induce cytokine production and expression of co-stimulatory molecules via the NF κ B signaling pathway (47, 48). AngII has been reported to up-regulate TLR4 levels in mouse mesangial cells (49), but there are not many studies that investigated a direct role of TLR4 on AngII-induced injury of renal tubular epithelial cells. Our data was consistent with the previous findings (29, 49) that AngII induced an increase in the expression of TLR4 in tubular epithelia. Further, we

silenced the gene for TLR4 using highly efficient siRNA to explore the specific effects of TLR4 in AngII-mediated hypertensive injury. We observed that suppression of TLR4 prevented the AngII-induced increase in the generation of TNF α and IL-1 β . Our results also showed that silencing of TLR4 attenuated the secretion of the inflammatory endogenous ligand, HMGB1, as evidenced by reduced gene and protein expression levels of HMGB1 in NRK52E cells. Since NF κ B activation is a downstream indicator of TLR4 activity, we measured the activity of NF κ B in these cells to confirm the role played by TLR4 signaling in kidney cellular injury induced by AngII. Our NF κ B activity data showed that there was an increased activation of NF κ B in NRK52E cells on AngII exposure. Interestingly, silencing of TLR4 attenuated the activation of NF κ B induced by AngII in these cell lines, thus validating our hypothesis that AngII mediated injury in renal tubular cells progress through the TLR4 signaling cascade.

TLR4 inflammatory signaling is initiated by the binding of a specific ligand of TLR4, when the receptor is in its proper functional conformation. HMGB1 is a well-known endogenous ligand of TLR4 and HMGB1 has the ability to trigger an inflammatory response (19-21). HMGB1-TLR4 interaction results in downstream activation of NF κ B, and subsequent inflammation via generation of PIC and reactive oxygen species (50). In addition to its ability to activate TLR4, HMGB1 by itself is an inflammatory protein (51, 52) released by immune cells and necrotic cells (16). Hence, there is interest in investigating the effects of HMGB1 neutralization in renal diseases. In an AngII-induced *in vitro* model of kidney injury, we studied the modulatory role played by HMGB1. We pretreated the NRK52E cells with neutralizing anti-HMGB1 before AngII exposure and observed that anti-HMGB1 could attenuate the inflammatory effects induced by AngII, thereby indicating a role for HMGB1 in the progression of inflammatory injury of the kidneys.

We have clearly shown that TLR4 and HMGB1 are elevated in renal tubular epithelial cells upon AngII treatment. Furthermore, we also found that suppression of either of these molecules attenuate the AngII-induced inflammation in these cells. There are no studies that have established a direct ligand-receptor relationship between AngII and TLR4. Recent studies have documented the paracrine effect of HMGB1 in lipotoxicity (53) and urothelial carcinoma cell lines (54). HMGB1 treatment is also reported to attribute paracrine activity to human fibroblasts (55). So a plausible explanation for this mechanism would be that the injurious effect of AngII on cells induces the release of HMGB1 into the extracellular milieu. This HMGB1, via paracrine signaling, activates TLR4 on the surface of nearby cells, inducing downstream activation of NFκB. Activation of NFκB induces gene transcription of PIC, which leads to further increase in ROS production, fostering a positive feedback mechanism, and eventually leading to renal injury. An illustration of the pathway that our data support is depicted in Figure 2.7A-B.

Limitations

In the present study, we have used the NRK52E cell line as our renal tubulo-epithelial cell model. Obviously, NRK52E cells may not exhibit the phenotypical characteristics of all kinds of cells in the kidney. However, NRK52E cells are widely used as a model to investigate AngII-mediated signaling (Yang F et al., 2013, Sanchez-Lopez E et al 2005) and redox mechanisms (Rice EK et al, 2003) in the kidneys. Therefore, we believe that the NRK52E cell line is an already validated renal tubulo-epithelial cell model for our study. Future studies involving other renal epithelial and immune cell types and *in vivo* experiments using animal models of AngII-induced kidney injury are required for further validation of this mechanism.

Another important limitation of this study is the involvement of specific AngII receptors in AngII-induced inflammatory injury observed in this study. Research studies suggest that the

Mechanisms by which TLR4-HMGB1 signaling contributes to the pathogenesis of AngII-induced hypertensive renal injury

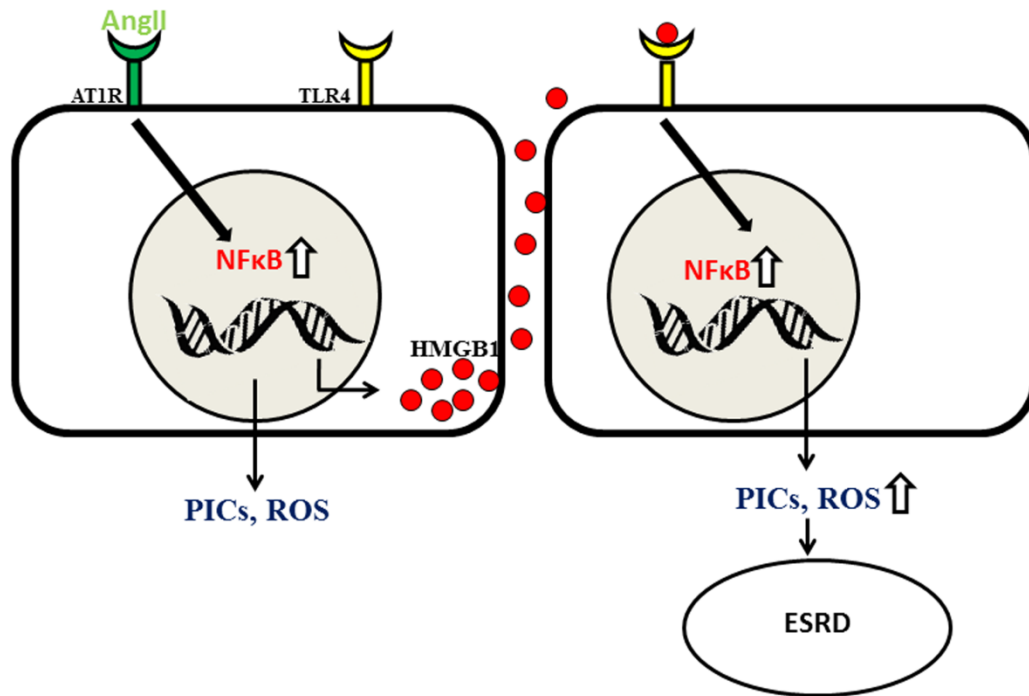


Figure 2.7A: A schematic showing the mechanisms by which TLR4-HMGB1 signaling contributes to the pathogenesis of AngII-induced hypertensive renal injury in rat tubulopithelial cells.

Proposed novel pathways by which AngII-mediated hypertensive end organ damage can be attenuated in rat tubulo-epithelial cells.

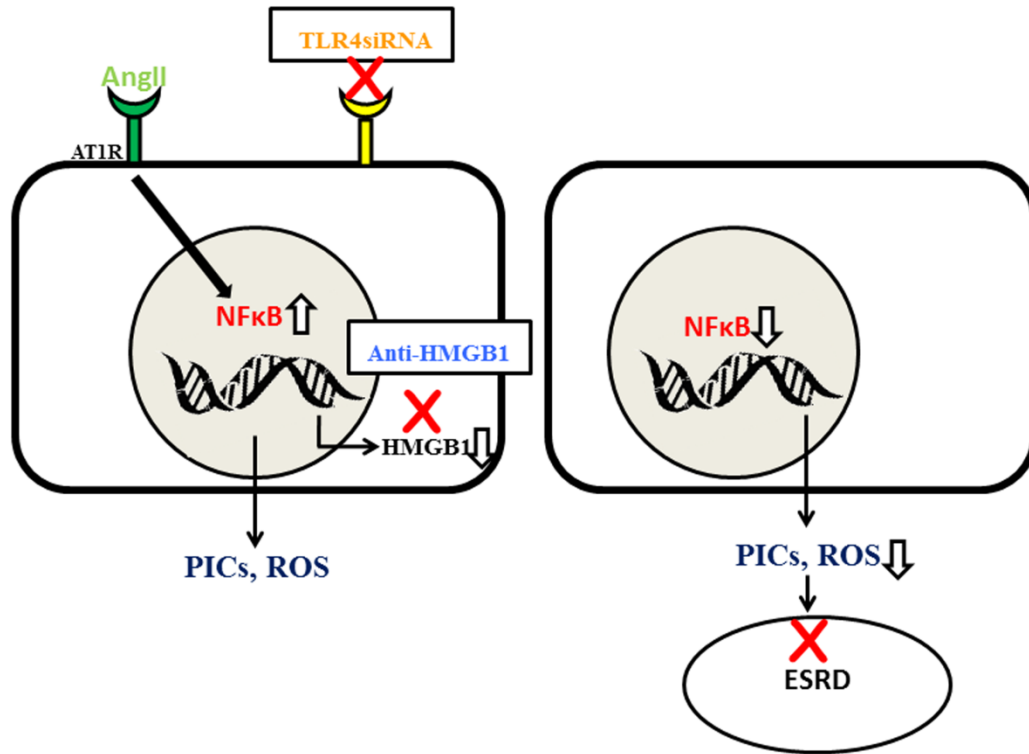


Figure 2.7B: A schematic showing the novel proposed pathways by which AngII-mediated hypertensive end organ damage can be attenuated in rat tubulo-epithelial cells.

proinflammatory effects of AngII are mediated by AngII type 1 receptor (AT1R). We have addressed this by pre-treating the cells with losartan. Our data show that losartan treatment caused a down-regulation of PICs in AngII exposed cells. Nevertheless, since there is limited evidence of a direct ligand-receptor relationship between AngII and TLR4, we cannot attribute the entire AngII-mediated renal injury to HMGB1-TLR4 signaling. Future *in vivo* studies involving an AT1R knock-out model is warranted to elucidate the comprehensive role of TLR4 signaling in AngII-induced renal injury.

HIGHLIGHTS:

- Ang II induced up-regulation of TLR4 inflammatory cascade in renal tubular cells
- AngII caused an increase in the expression of HMGB1 in NRK52E cells
- Increased HMGB1-TLR4 signaling induced subsequent inflammation and PIC production
- Blockade of TLR4 and HMGB1 attenuated AngII induced inflammation in NRK52E cells
- HMGB1-TLR4 signaling, a potential pharmacological target in inflammatory renal diseases

ACKNOWLEDGEMENTS:

The authors gratefully acknowledge Ms. Marilyn Dietrich for her help with Flow cytometry.

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CHAPTER 3

ROLE OF TLR4 IN LIPOPOLYSACCHARIDE-INDUCED ACUTE KIDNEY INJURY: PROTECTION BY BLUEBERRY

- This chapter previously appeared as Anand R. Nair, Gustavo S. Masson, Philip J. Ebenezer, Fabio Del Piero, and Joseph Francis. Role of TLR4 in Lipopolysaccharide-induced Acute Kidney Injury: Protection by Blueberry, *Free Rad Biol Med* 2014 Jun;71:16-25. doi: 10.1016/j.freeradbiomed.2014.03.012. It is reprinted by permission of Elsevier – see the permission letter for proper acknowledgement phrase.

3.1 INTRODUCTION

Acute kidney injury (AKI) is a significant cause of morbidity and mortality in patients[1], particularly affected by trauma[2]. In addition to the sudden tubular and endothelial cell injury, AKI also involves other detrimental effects like apoptosis, intra-luminal obstruction and necrosis [3]. Previous studies indicate that the oxidative stress-induced inflammatory cascade causes the renal cellular injury in acute renal failure[4]. Hence, there is a need for better strategies to attenuate the inflammation that is associated with such conditions, which could also be a promising therapy.

Inflammation has been implicated in most of the kidney-related disorders, including nephropathy [5], hypertension and renal failure. Inflammation triggered by the excessive production of proinflammatory cytokines (PIC) has been characterized in hypertension-induced renal injury [6]. PIC have been shown to exacerbate ROS generation [7, 8], which can activate several intracellular signaling pathways including the one that involves the transcription factor NFκB [9, 10]. The NFκB signaling pathway further activates the production of more PIC, which in turn increases the ROS generation and therefore gives rise to a vicious cycle. The contribution

of Toll-like receptor 4 (TLR4) is of particular interest as it is an important molecule that mediates several inflammatory pathways.

TLRs are receptors found on the cell surface, which play a key role in the innate immune system. TLRs recognize pathogens or foreign molecules and trigger the innate immune response and are shown to induce cytokine production and expression of co-stimulatory molecules [11] via the NF κ B signaling pathway [12, 13]. Another interesting aspect of TLR4 is that it serves as the specific ligand for the binding of lipopolysaccharide (LPS), which is the major cell wall component of gram-negative bacteria. Incidentally, the TLR4 agonist LPS also causes sepsis and acute kidney injury and has been well studied [14]. It has been shown that LPS induced sepsis causes hypotension, vascular damage, and reduced tissue perfusion leading to end organ damage [15]. Again, the TLR4-specific viral inhibitory peptide VIPER has been shown to potentially inhibit TLR4-mediated responses induced by LPS [16]. Taken together, TLR4 is a key molecule that could mediate the NF κ B inflammatory cascade leading to AKI.

Blueberries (BB) are a rich source of antioxidants and known to have very high anti-inflammatory properties. Studies have previously depicted the beneficial effects of BB in lowering mean arterial pressure in hypertensive rats [5], reducing the production of PIC in rat glial cells [17] and protecting the kidney from nephropathy [5]. Therefore, we hypothesize that BB protect against AKI by modulating TLR4. To test our hypothesis, we used LPS, a specific ligand for TLR4, to induce AKI. In addition, we determined whether BB protect against AKI by inhibiting TLR4 and compared the reno-protective effects of BB with VIPER, a specific TLR4 inhibitor.

3.2 MATERIALS AND METHODS

Ethics Statement

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 (protocol approval number 12-007).

Animals and Experimental design

Twelve-week old Sprague-Dawley rats from colonies maintained in the Louisiana State University School of Veterinary Medicine were used in this study. The original source of breeder animals was Harlan (Indianapolis). All animals were maintained on normal rat chow (ad-libitum) and water, housed in a temperature- ($23\pm 2^{\circ}\text{C}$), humidity- and light- (12 hour light/dark cycle) controlled environment.

Six groups of eight animals each were used for the experimental protocol: Saline fed control (SAL+Cont), BB fed Control (BB+Cont), VIPER-treated Control (VIPER+Cont), saline fed-LPS injected (SAL+LPS), BB fed-LPS injected (BB+LPS), VIPER and LPS injected (VIPER+LPS). The saline and BB feeding was done for 2 days, once both day (A pilot study was conducted to determine the appropriate BB and VIPER doses. Rats were fed with BB for 1 or 2 days and it was found that 2 day BB feeding was protective). The animals were injected with LPS (Sigma Aldrich L3129; 10mg/kg bw i.p) 6 h before performing the acute renal clearance experiments. The injections were given at 8 AM in order to minimize the light-dark cycle variations. The rats belonging to the VIPER+LPS group were administered with VIPER

(0.1mg/kg i.v) 2 h prior to the LPS injection. Rats were euthanized at the end of the acute renal clearance experiments and kidneys were obtained for analyses.

Diets

The food consumption was measured daily for a week prior to the start of the study. The BB powder was obtained from the United States Highbush Blueberry Council (USHBC). The BB powder (T10711) is a 50/50 blend of two varieties, Tifblue and Rubel. To prepare the 2% BB solution, the BB powder was homogenized in water and filtered. Each rat was supplemented with BB equal to 2% of their daily feed intake. This solution was fed to animals by an intra-gastric gavage.

Acute renal clearance experiments

Rats from all the study groups were subjected to renal clearance experiments at the end of the feeding period as described previously [5, 18]. Briefly, each rat was anesthetized with Inactin (thiobutabarbital ; 100mg/kg body weight), the right inguinal area was shaved, a small incision made, and femoral vessels were carefully isolated. The femoral artery was cannulated with heparin-primed (100U/ml) PE-50 polyethylene tubing connected to a pressure transducer (PowerLab data acquisition systems; AD Instruments, Colorado Springs, CO) for continuous measurement of arterial pressure. The femoral vein was catheterized with heparin-primed PE-50 tubing for infusion of solutions at 20 μ l/min. An isotonic saline solution containing 6% albumin was infused during surgery. After surgery, the infusion solution was replaced with isotonic saline containing 2% bovine serum albumin (BSA), 7.5% inulin (Inutest) and 1.5% PAH, and a 300 μ l bolus of this solution was administered at the start of each clearance experiment. The bladder was exposed via a suprapubic incision and catheterized with a PE-160 tube for gravimetric urine collection. After a 15-20 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 the glomerular filtration rate (GFR) and renal plasma flow (RPF), respectively.

Electron paramagnetic resonance (EPR) spectroscopy

Total ROS and superoxide production rates were measured in kidney cortex and medulla as previously described [5, 18-20] and in whole blood. The '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. In addition to EPR, there are other reported analytical approaches like the use of chemiluminescent and fluorescent probes to measure reactive oxygen and nitrogen species, but all have their merits and limitations[21]. All electron paramagnetic resonance (EPR) spectroscopy measurements were performed with a benchtop EPR spectrophotometer e-scan R (Noxygen Science Transfer and Diagnostics, Elzach, Germany). 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) was used to measure total ROS and superoxide in whole blood and kidney tissues.

Measurement of ROS and superoxide in kidney tissues - Pieces of kidney tissue were incubated at 37⁰C with 6.6µl of CMH (200µM) for 30 min for ROS measurement; CMH for 30 min, then 1.5µl of PEG-SOD (50U/ml) for an additional 30 min for superoxide measurement. Aliquots of incubated probe media were then taken in 50µl disposable glass capillary tubes (Noxygen Science Transfer and Diagnostics) for determination of total cortical ROS and superoxide production, under the EPR settings as described previously[9, 20, 22].

Measurement of ROS and superoxide in whole blood – Whole blood (20µl) was added to a tube containing 20µl of CMH (400µM), mixed gently for ROS measurement. Superoxide was measured by adding 20µl of whole blood to a tube containing 20µl of CMH (400µM) and then adding 0.64µl of PEG (50U/ml). In both cases, the samples were taken in 50µl disposable glass capillary tubes (Noxygen Science Transfer and Diagnostics) and measured on the EPR instrument.

RNA extraction and Real-Time PCR

Real-time PCR was used to determine the mRNA expression levels [22-24] of the kidney cortical (KC) proinflammatory cytokines (PIC), TLR4 and AKI markers by using specific primers. Total RNA was isolated using Trizol Reagent (Invitrogen, CA). The RNA concentration was calculated from the absorbance at 260nm and RNA quality was assured by the 260/280 ratio. The RNA samples were treated with DNase I (Ambion) to remove any genomic DNA. First strand cDNA were synthesized from 2µg RNA with an iScript cDNA synthesis kit (Bio-rad, Hercules, CA). Real-Time PCR was performed in 384-well PCR plates using iTaq SYBR Green Super mix with ROX (Bio-rad) in triplicate using the ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA). The PCR cycling conditions were as follows: 50°C for 2 mins, 95°C for 3 min, followed for 45 cycles (15 s at 95°C and 1 min at 60°C). To confirm the specific PCR product, a dissociation step (15 s at 95°C, 15 s at 60°C and 15 s at 95°C) was added to check the melting temperature. Gene expression was measured by the $\Delta\Delta CT$ method and was normalized to GAPDH or 18s RNA mRNA levels. The data presented are the fold changes of the gene of interest relative to that of the control animals.

Western Blotting analysis

Primary rat TLR4 (SC-30002, Santa Cruz Biotechnology, Inc), KIM-1 (ab-47634, Abcam, USA), TNF- α (ab-66579, Abcam, USA) antibodies were used following manufacturer-recommended dilutions, followed by a horseradish peroxidase-conjugated anti-rabbit IgG antibody (SC-2004, Santa Cruz Biotechnology, Inc). Rat primary anti-actin or anti-GAPDH was used to confirm the loading and the transfer. We used ImageJ software to analyze the bands.

Immunofluorescence detection

Rat tissues were prepared for immunofluorescence as previously described [25, 26]. Sequential kidney coronal sections (30 μ) were cut with a cryostat (Leica CM 1850; Nussloch, Germany) and were collected in tissue culture wells with 0.1M phosphate buffer. Free-floating sections were incubated with 2% normal goat serum for 30 min in phosphate buffer (0.1M). Mouse anti-TLR4 antibody (1:500 dilution; ab-22048, Abcam, USA) and rabbit anti-KIM-1 (1:1000 dilution; ab-47634, Abcam, USA) were used for immunofluorescence staining for 24 hours at 4°C. The primary antibodies were diluted in 0.1M phosphate buffer with 1% normal goat serum and 0.1% Triton-X. Slices were washed 5-times with phosphate buffer (0.1M) and incubated with goat anti-mouse Alexa 594-conjugated antibody (1:1000 dilution, Invitrogen, USA) and goat anti-rabbit Alexa 488-conjugated antibody (1:1000 dilution, Invitrogen, USA) for 1 h at room temperature. After secondary antibody incubation, slices were washed 3 times with phosphate buffer (0.1M) and 6 times with Tris HCl (1M). Four slices were placed on each slide and mounted with a coverslip.

The histological sections were carefully examined under a fluorescence microscope (IX71 Olympus, USA). The images of the kidney sections, with tubules and glomeruli, were

digitized at 20x using Olympus DP2-BSW software. Tissue endogenous fluorescence was not found at microscope settings which were used to analyze the slide.

Quantification of NFκB p65 activity

The NFκB/p65 activity ELISA (Active Motif, USA) kit was used to assess the binding activity of free NFκB p65 in nuclear extracts, as described previously [23, 27]. A sandwich ELISA method was employed to perform the analysis, according to the manufacturer's instructions.

3.3 RESULTS

BB preserves renal hemodynamics in LPS-induced AKI model

Renal hemodynamic changes are sequelae of AKI and LPS is known to induce a loss of function of the kidney, thus contributing to the development of AKI. The kidney function in rats from all study groups was assessed to determine the extent of kidney damage. The mean arterial pressure (MAP) was not significantly different between control and experimental groups (Fig 3.1 A).

However, the hypotensive effect of LPS was evident in the SAL+LPS rats, where the MAP decreased when compared to the controls. BB+LPS treatment showed a tendency to improve the MAP, but were not statistically significant. The renal clearance experiment showed that there was a significant reduction in GFR and RBF and an increase in RVR in SAL+LPS animals. In contrast, in the BB+LPS group, there was significant improvement in GFR, RBF and a decrease in RVR indicating a renoprotective effect of BB in AKI. Interestingly, we also observed an attenuation of renal function parameters in VIPER+LPS animals, indicating a specific role of TLR4, at least in part, in the functional decline in the kidneys of LPS-induced AKI animals. However, no hemodynamic changes were observed between SAL+Cont,

VIPER+Cont (data not shown) and BB+Cont groups (Fig 3.1). These results indicate that BB has a protective effect against the renal dysfunction developed in LPS-induced AKI rats.

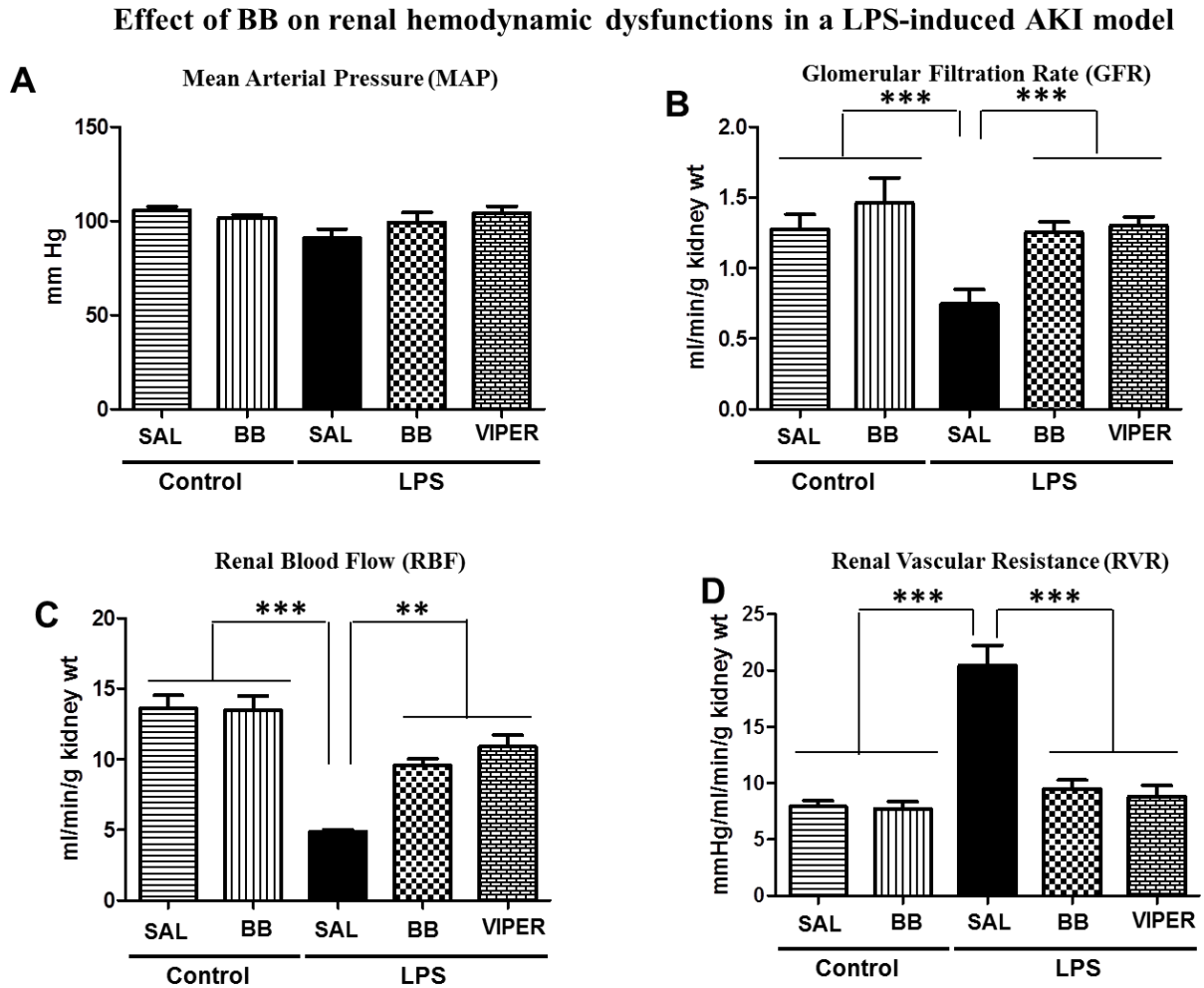


Figure 3.1: Effect of BB on renal hemodynamic dysfunctions in a LPS-induced AKI model. The kidney function of rats from all experimental groups as assessed by renal clearance experiments (n=6-8 per group) All values are presented as mean \pm SEM (**p < 0.01, ***p < 0.001)

BB inhibits the generation of free radicals in the kidney and whole blood of LPS-induced AKI rats

We measured the rate of generation of free radicals (ROS and superoxide), both tissue level (Table 3.1) and circulating (Table 3.2), in animals from all experimental groups. At the tissue level, both in kidney cortex and kidney medulla, the production of total ROS and superoxide were significantly increased in the SAL+LPS group compared to the rats from SAL+Cont, VIPER+Cont (data not shown) and BB+Cont groups. Nevertheless, BB treatment attenuated the rate of cortical and medullary free radical production in the BB+LPS animals. Animals pretreated with VIPER also, upon LPS administration, had a reduction in the generation of these free radicals in the kidney tissue.

The circulating free radical production was measured in whole blood at the beginning (baseline) and at the end of the study (final). Baseline rates of production of total ROS and superoxide were not different among the groups. The total ROS and superoxide generation, however at the end of the study, was significantly elevated in animals treated with LPS (SAL+LPS) compared to their control counterparts. In contrast, animals pre-treated with BB or VIPER and injected with LPS (BB+LPS; VIPER+LPS) had a significantly decreased total ROS and superoxide in the blood. These results signify that BB exerts an anti-oxidant effect on LPS-treated AKI model.

Table 3.1: Total ROS and superoxide production in the renal cortical and medullary tissue. LPS treatment induced an increase in the generation of both total ROS and superoxide in the kidney cortex (n=6-8) and medulla (n=6-8). Significantly reduced total ROS and superoxide production was noted in the rats pretreated with BB before an LPS insult. VIPER treatment also attenuated the total ROS and superoxide production. All values are presented as mean \pm SEM (*- $p < 0.05$ vs.SAL+Cont, # - $p < 0.05$ vs. SAL+LPS ,\$- $p < 0.05$ vs. BB+LPS)

	Control		LPS		
	SAL	BB	SAL	BB	VIPER
Renal Cortex					
Total ROS (μ M/mg protein/min)	0.115\pm0.009[#]\$	0.119\pm0.021^{\$}	0.329\pm0.059^{*\$}	0.204\pm0.024[#]	0.211\pm0.22[#]
Superoxide (μ M/mg protein/min)	0.021\pm0.005[#]	0.023\pm0.004	0.068\pm0.019^{*\$}	0.021\pm0.004[#]	0.019\pm0.006[#]
Renal Medulla					
Total ROS (μ M/mg protein/min)	0.141\pm0.036[#]\$	0.149\pm0.030^{\$}	0.293\pm.051^{*\$&}	0.218\pm0.023[#]	0.201\pm0.035[#]
Superoxide (μ M/mg protein/min)	0.029\pm0.009[#]	0.026\pm0.003	0.042\pm0.009^{*\$&}	0.027\pm0.004[#]	0.022\pm0.009[#]

Table 3.2: Total ROS and superoxide production in the whole blood at baseline and end of the study. At baseline (n=6-8), there was no significant difference between the rate of total ROS and superoxide production in rats from all experimental groups. However, at the end of the study, the LPS treated animals showed a significantly higher rate of total ROS and superoxide generation. Pretreatment with BB and VIPER was able to inhibit the increase in the rate of free radical production. All values are presented as mean \pm SEM (*- p < 0.05 vs. SAL+Cont, # - p < 0.05 vs. SAL+LPS, \$- p < 0.05 vs. BB+LPS)

	Control		LPS		
	SAL	BB	SAL	BB	VIPER
Baseline					
Total ROS (μ M/mg protein/min)	1.527 \pm 0.123	1.641 \pm 0.109	1.600 \pm 0.150	1.591 \pm 0.121	1.613 \pm 0.131
Superoxide (μ M/mg protein/min)	0.170 \pm 0.038	0.158 \pm 0.048	0.152 \pm 0.035	0.153 \pm 0.023	0.162 \pm 0.020
Final					
Total ROS (μ M/mg protein/min)	1.510 \pm 0.088 [#]	1.591 \pm 0.086 ^{\$&}	1.959 \pm 0.171 ^{*\$}	1.420 \pm 0.079 [#]	1.533 \pm 0.092 [#]
Superoxide (μ M/mg protein/min)	0.182 \pm 0.023 [#]	0.165 \pm 0.040 ^{\$&}	0.457 \pm 0.187 ^{*\$}	0.262 \pm 0.061 [*]	0.241 \pm 0.050 ^{#*}

BB decreases the expression of TLR4 in the renal cortical tissues of LPS-induced AKI rats

Since it is well established that LPS is a direct ligand of TLR4, we measured the cortical gene and protein expression levels of TLR4 in all experimental groups at the completion of the study (Figure 3.2). Both gene and protein expression of TLR4 was elevated in LPS-induced AKI rats (Sal+LPS) compared to the saline, VIPER and BB treated controls (SAL+Cont, VIPER+Cont, BB+Cont), indicating that the increased inflammation in the kidney is at least in part due to TLR4 activation in these animals. In contrast, pretreatment with BB prevented TLR4 activation, confirming that renoprotective effect is, at least in part, mediated by TLR4. The TLR4 expression was however normalized in BB+LPS rats. The decreased protein expression of TLR4 in VIPER+LPS group also confirms the role of TLR4 in mediating LPS-induced renal injury.

BB attenuates NF- κ B activity and proinflammatory cytokine expression in the kidney of LPS-induced AKI animals

The activation of TLR4 was confirmed by measuring the NF κ B activity. We measured the renal cortical NF κ B p65 DNA binding activity in tissues from saline and BB treated groups (n=7). Consistent with the TLR4 expression results, rats from SAL+LPS group had significantly higher cortical NF κ B activity compared to SAL+Cont and BB+Cont groups (Fig 3.3A). The BB+LPS rats had a significantly lower NF κ B activity levels compared to the SAL+LPS rats suggesting that the BB supplementation prevents the increase in NF κ B activity seen in LPS-induced AKI rats. The rats that were pretreated with VIPER (VIPER+LPS) also exhibited an attenuated NF κ B activity.

Effect of BB on renal TLR4 gene and protein expression in a LPS-induced AKI model

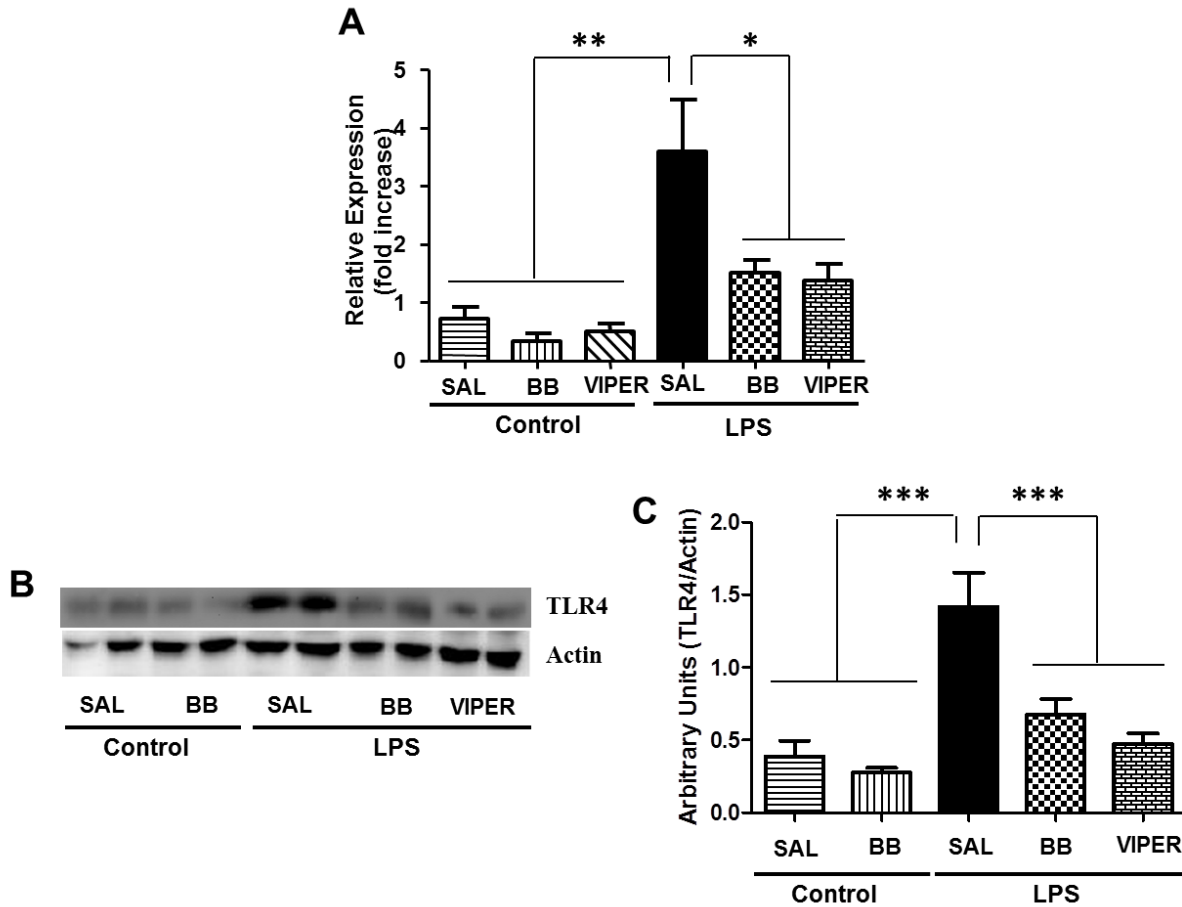


Figure 3.2: Effect of BB on renal TLR4 gene and protein expression in a LPS-induced AKI model. The (A) mRNA (n=6) and (B) a representative western blot of TLR4 in the renal cortical tissues. (C) Bands were analyzed and quantified by densitometry (n=6). The gene and protein expression of TLR4 was increased in the LPS treated rats. The pretreatment with BB was able to inhibit the increased expression of TLR4 in LPS treated animals and was comparable to VIPER-pretreated rats. All values are presented as mean \pm SEM (* p < 0.05, ** p < 0.01, *** p < 0.001)

Effect of BB on the renal inflammatory profile in a LPS induced AKI model

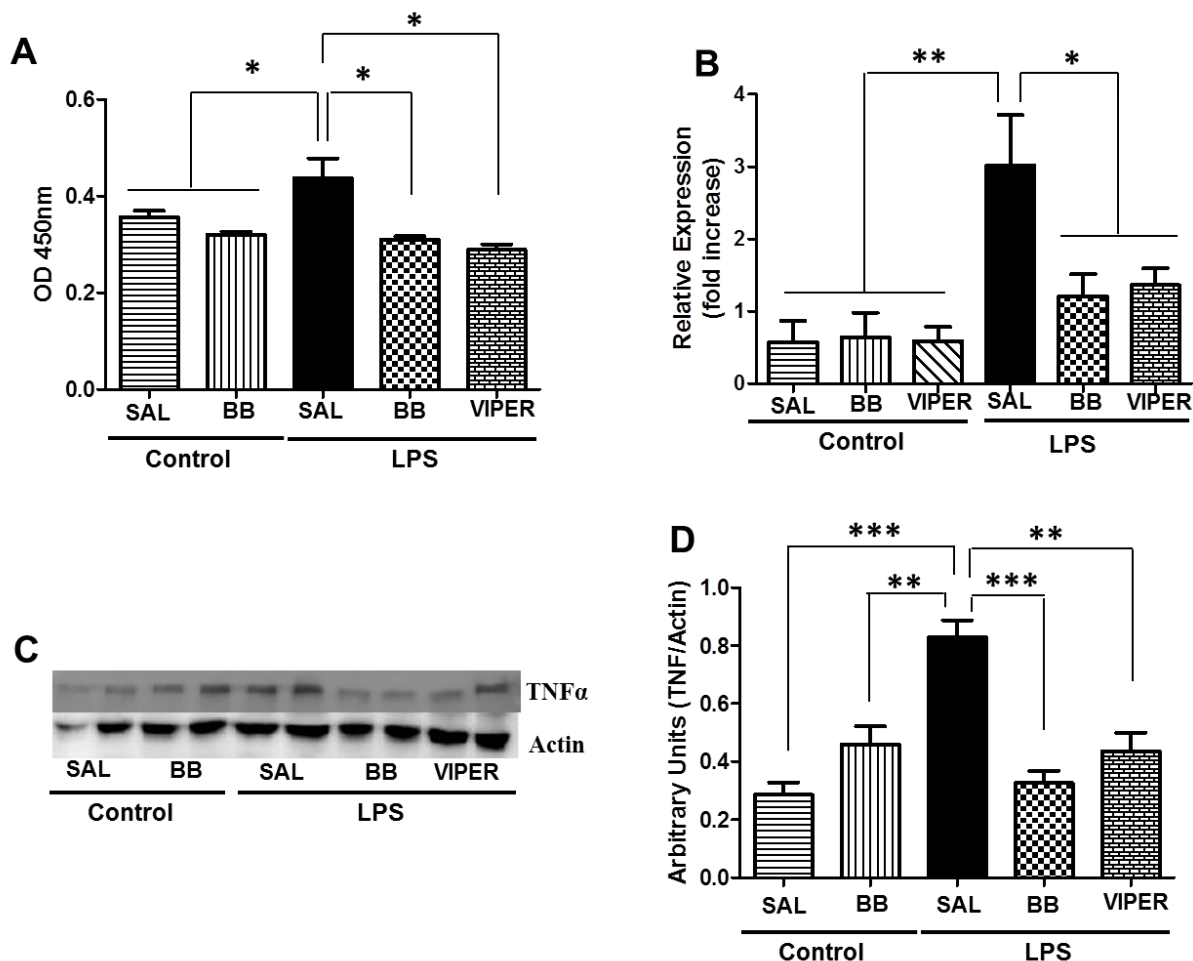


Figure 3.3: Effect of BB on the renal inflammatory profile in a LPS induced AKI model. (A) DNA binding activity of NFκB p65 subunit in renal cortical tissues of rats from each experimental group (n=6), as determined by ELISA. Increased NFκB p65 DNA binding activity in LPS treated animals was significantly reduced by BB treatment. The (B) mRNA expression (n=6) of TNFα in the renal cortical tissues. (C) A representative western blot showing TNFα protein expression. (D) Western blot bands were analyzed and quantified by densitometry. TNFα mRNA and protein expression (n=6) were increased in LPS treated group and this was attenuated by pretreatment with BB. The TNFα protein expression in the BB + LPS group was comparable to the VIPER+LPS rats (*p < 0.05, **p < 0.01)

The expression levels of tumor necrosis factor- α (TNF- α) in the renal cortical tissues of animals from all experimental groups were measured (Figure 3.3B, C and D) since TNF- α predominantly acts via the NF κ B dependent pathway. Expression of TNF- α was significantly increased in SAL+LPS rats compared to the SAL+Cont, BB+Cont and VIPER+Cont rats. However, the levels of TNF- α in BB+LPS and VIPER+LPS rats were comparable to the SAL+Cont and BB+Cont rats.

BB ameliorates the renal damage in LPS-induced AKI rats

The acute kidney injury caused by the LPS was confirmed by measuring the expression levels of acute kidney injury markers. The gene expression levels of KIM-1 (Fig 3.4A), cystatin C (Fig 3.4D) and IL-18 (Fig 3.4E), all of which are markers of AKI, and protein expression of KIM-1 (Fig 3.4B, 3.4C) were determined. Compared to control groups, KIM-1, cystatin-C and IL-18 expression was increased in rats of SAL+LPS group but were considerably lower in BB+LPS rats. These data suggest that BB supplementation has a protective role in AKI as indicated by decreased renal injury markers. The protein expression levels of KIM-1 in animals pretreated with VIPER were also assessed in order to investigate the role of TLR4 in the progression of renal injury and our data (Fig 3.4B, 3.4C) showed that VIPER treated rats had an attenuated KIM-1 protein expression.

The beneficial effects of BB in the kidneys is, at least in part, TLR4-dependent/ TLR4 and KIM-1 cell type localization in the LPS-induced kidney injury

We performed immunofluorescent double-staining on the kidney to determine if there is co-localization of TLR4 and KIM1 in renal tissues. We observed that TLR4 and KIM-1 were activated in both glomerular and tubular cells of SAL+LPS animals. We were also able to identify TLR4 and KIM-1 co-localization in these animals.

Effects of BB on acute renal injury markers in a LPS induced AKI model

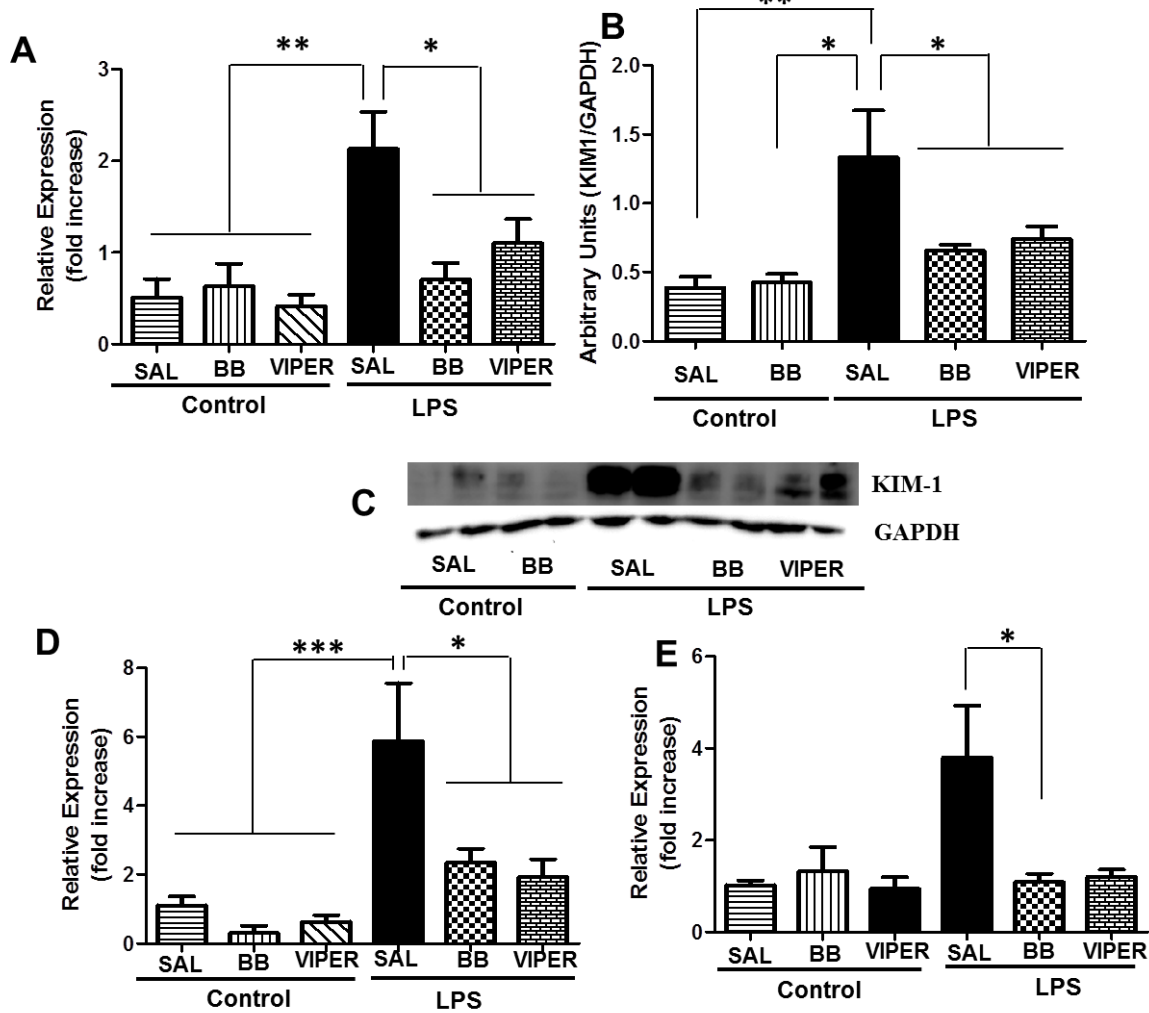


Figure 3.4: Effects of BB on acute renal injury markers in a LPS induced AKI model. The expression levels of biological AKI markers were assessed in all experimental groups. The (A) mRNA (n=6) and (B) protein expression (n=6-8) of KIM1 was increased in the kidney of LPS treated animals. BB and VIPER pretreatment reduced the expression of KIM1 in renal cortical tissue. (C) A representative western blot showing KIM1 expression in rats from all study groups. The mRNA expression of acute renal injury markers (D) Cystatin-C (n=6) and (E) IL-18(n=6). (*p < 0.05, **p < 0.01)

Consistent with our mRNA and western blot results, SAL+LPS animals had markedly increased TLR4 and KIM-1 expression in the kidneys, compared to the SAL+Cont and BB+Cont groups. The BB+LPS and VIPER+LPS animals, however, had a significantly reduced expression of TLR4 and KIM-1 in both glomeruli and tubules, and were comparable to the controls. This data indicates an autocrine effect of the TLR4 pathway in activating the inflammatory cascade within a cell and inducing cellular injury, thus resulting in the activation of kidney injury markers. Treatment with BB could inhibit this TLR4 mediated expression of kidney injury marker KIM-1 (Figure 3.5).

3.4 DISCUSSION

The salient finding of this study is that BB protect against renal injury by inhibiting TLR4 expression. In addition, BB, by inhibiting TLR4, also improves renal function, thereby indicating a direct effect of TLR4 in modulating inflammation in the kidney and renal hemodynamics. Most TLR, including TLR4, are known to activate the NF κ B inflammatory cascade [28,29]. Here, we also show that BB attenuates the NF κ B activity, thus confirming the renoprotective effect of BB is, at least in part, mediated by TLR4. We also used VIPER, which is a TLR4 specific inhibitory peptide, to substantiate the protective effects of BB.

Blueberries are a rich source of antioxidants and are known to have antioxidant and anti-inflammatory properties. BB are known to contain active antioxidant polyphenols, such as anthocyanins, flavanols and phenolic acid. Recent studies have revealed that an anthocyanin-rich diet can reduce the risk of myocardial infarction in women [30] and indicated that anthocyanins could be a potential inhibitors of inflammation [31]. Our lab and others have previously shown

Effects of BB on KIM1 and TLR4 expression in renal tissue

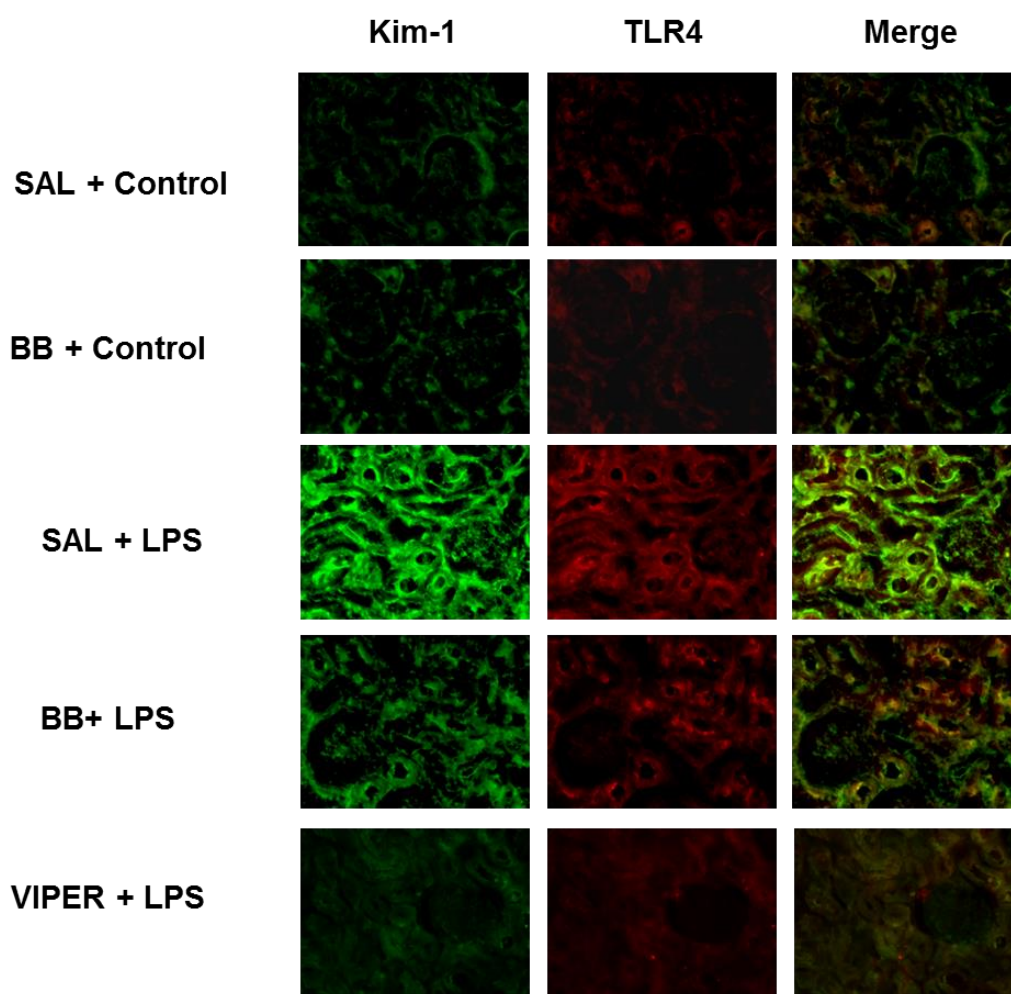


Figure 3.5: Effects of BB on KIM1 and TLR4 expression in renal tissue. Representative immunofluorescence images for TLR4 and KIM1 staining in kidney sections from rats from all experimental groups. We identified expression of TLR4 and KIM1 in glomeruli and tubules, which was dramatically increased on LPS treatment. Interestingly, BB pretreatment, similar to VIPER treatment, seems to decrease the staining of TLR4 and KIM1 in the glomeruli and tubules. Double-labelled staining revealed a colocalization of TLR4 and KIM1.

the beneficial effects of BB in attenuating blood pressure [32] and thus exerting a protective effect against hypertension-induced nephropathy in spontaneously hypertensive rats [5]. Even though several studies have documented the antioxidant and anti-inflammatory properties of BB, precise molecular mechanism by which BB protects against inflammation and inflammation-associated diseases is not clear. This study was designed to investigate if BB could protect against an acute renal injury by modulating TLR4. Here we show for the first time that BB treatment attenuates the TLR4 expression in the renal tissue of LPS-induced AKI animals. Since we used LPS (a specific ligand for TLR4) to induce AKI and its effect was blocked by BB. This result indicate that the renoprotective effects BB are, at least in part, mediated by the inhibition of TLR4.

AKI is characterized by a sudden loss of function of the kidneys, thus disturbing the body fluid homeostasis [33]. It remains a major cause of mortality in Western society and increases the risk of end stage renal disease [34, 35]. The most effective way to avoid the development of end stage renal disease is to prevent the incidence of AKI or delay its progression. Pharmacological therapeutics available for AKI currently have been successful to an extent, but there has been an increasing interest in non-pharmacological approaches to treat acute and chronic renal diseases. One such non-pharmacological therapeutic method is the use of natural sources of antioxidants, such as blueberries.

AKI may be a consequence of trauma [2], ischemic injury or a nephrotoxic insult [33]. A well-known nephrotoxic agent is LPS (lipopolysaccharide), which is an endotoxin that is found on the cell wall of gram-negative bacteria. LPS, a specific ligand for the TLR4 receptor, has been used in several animal models to induce septic shock, which is characterized by an inflammatory response [36] leading to AKI [37]. Recent studies have shown that the LPS-induced

inflammation [38] and renal tubule impairment associated with sepsis [39] are through the activation of TLR4. Mice lacking the gene for TLR4, upon ischemia-reperfusion of the kidney, had decreased production of proinflammatory cytokines and macrophage infiltration [40]. In this study, we used LPS to induce kidney dysfunction in rats. Our findings indicate that the LPS-treated rats showed a marked decrease in kidney function as evidenced by a reduction in GFR and RBF, along with an increase in RVR. These results are consistent with previous findings [41-43]. It has been shown that LPS progressively increases RVR in rats even at minimal doses [41, 44].

In the kidneys, LPS insult has been shown to induce oxidative stress, activating generation of ROS [45] and other free radicals [46]. Superoxide anions are known to induce an increase in smooth muscle cell contraction by regulating the cytosolic calcium concentration [47]. Our EPR data indicate that the rate of generation of total ROS and superoxide in the LPS-treated animals was increased significantly in the kidney as well as in the whole blood. Therefore, this increase in the key mediators of systemic as well as renal oxidative stress can cause vasoconstriction of the arterioles [48], thus contributing to the reduction and elevation in the RBF and RVR respectively.

An increase in RVR has been associated with hypoxia in the kidneys [49]. Under hypoxic conditions, the tissue is at a greater risk for injury [50-52], wherein it develops glomerular and tubular injury, accompanied by a loss of nephrons. In addition to the hypoxia caused by the vascular resistance, LPS has also been shown to induce glomerular and tubulo-interstitial damage in the renal tissue [53, 54]. For instance, LPS has been shown to cause ultra-structural alterations in the glomerular endothelium [53]. LPS treated mice were shown to have a damaged glomerular endothelial surface layer and a 5-fold decrease in the density of the glomerular

endothelial fenestrae [53]. This damage to the glomerular endothelium can impede the proper filtration of the fluid by the glomerulum. Further, studies have associated KIM-1, a tubular injury marker in AKI, with low GFR levels [55]. KIM-1 is a tubular trans-membrane glycoprotein that is stimulated after an ischemia-reperfusion or a nephrotoxic insult [56]. Therefore, in the light of these reports and our current findings, we can propose that the glomerular and tubulo-interstitial injury induced by the LPS is the possible cause of the reduced GFR in these animals. Interestingly, the rats from BB+LPS group had significantly improved GFR and RBF, and reduced RVR indicating the ability of BB in protecting against oxidative stress, thereby down-regulating TLR4 levels, hence underlining the role of TLR4 in improved hemodynamics and reno-protection.

In addition to KIM-1 [57, 58], AKI involves upregulation of other biological injury markers like IL-18 [59] and cystatin-C [60]. We measured gene expression levels of these AKI markers and protein expression of KIM-1 in the cortical kidney tissues of these rats. Our findings indicate that the gene and protein expression of these AKI markers in the kidney in BB+LPS was significantly lowered when compared to the SAL+LPS group. Our immunofluorescence data indicates a co-localization of TLR4 and KIM-1 in the kidney of LPS-treated rats. As LPS is the direct ligand of TLR4, an LPS insult would activate the TLR4 pathway. This activated TLR4 pathway could possibly have an autocrine effect in the kidney cells, wherein it triggers an inflammatory cascade and thereby contributes to cellular injury. This injury caused to the cell would elicit the production of KIM-1 and hence result in the increased expression of both TLR4 and KIM-1 in the same cell. This vicious cycle of increased oxidative stress, TLR4 activation and KIM1 will further exacerbate renal injury resulting in the decline in renal function. BB by its ability to attenuate oxidative stress and prevent the TLR4 activation, blocks the vicious cycle and

its subsequent effect on renal injury, resulting in the restoration of renal function in this AKI model.

In addition to an attenuated TLR4 expression, BB+LPS exhibited decreased expression of PICs. We examined the expression of TNF α in the kidney cortex tissue of animals from all experimental groups. We observed that BB+LPS animals expressed a significantly lesser amount of TNF α compared to the SAL+LPS rats. This is consistent with the previous findings from our lab, which reported that in SHR, a renal function decline involves an association of PICs with their transcription factor NF κ B [9]. Further, oxidative stress is a key regulator of NF κ B activity [10] and it produces a positive feedback mechanism related to inflammation and tissue injury. In the present study, blueberry was able to decrease oxidative stress induced by an acute LPS insult and this antioxidant effect could be responsible for the NF κ B inhibition and decreased TLR4 expression.

Having discussed these novel findings of our study, we would also shed light on the possible limitations of this study. Firstly, even though we report that the renoprotective effect of BB in a LPS-induced AKI model is, at least in part, through TLR4 inhibition, there could be other plausible mechanisms that might contribute to the LPS-induced kidney injury and also mediate the renoprotective effects of BB. Secondly, at this time we do not know which particular component in BB is largely responsible for the renoprotective effect shown in this model.

In summary, our results suggest a major role of BB in protecting against LPS-induced AKI via a decrease in inflammation and most importantly, this study provides comparative evidence for the first time that the mechanism of action of BB is similar to VIPER, via inhibiting TLR4 expression. This study also suggests that non-pharmacological antioxidant approaches have protective value against renal injury and also divulge a potential target in TLR4 against

acute renal injury. Further studies are underway to determine the prospects of natural antioxidant sources not just as a preventative, but also as a therapeutic in renal diseases.

3.5 REFERENCES

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CHAPTER 4

A BLUEBERRY-ENRICHED DIET IMPROVES RENAL FUNCTION AND REDUCES OXIDATIVE STRESS IN METABOLIC SYNDROME ANIMALS: POTENTIAL MECHANISM OF TLR4-MAPK SIGNALING PATHWAY

- This chapter previously appeared as Anand R. Nair, Carrie M. Elks, Jorge Vila, Fabio Del Piero, Daniel B. Paulsen and Joseph Francis. A blueberry-enriched diet improves renal function and reduces oxidative stress in Metabolic syndrome animals: potential mechanism of TLR4-MAPK signaling pathway, *PLoS One*. 2014 Nov 5;9 (11):e111976. doi: 10.1371/journal.pone.0111976. Open access article, no permission required.

4.1 INTRODUCTION

Metabolic syndrome (MetS) is characterized by a cluster of health factors that indicate a higher risk for renal dysfunction and cardiac diseases. The prevalence of MetS in the United States is increasing at an alarming pace, 34% of adults being affected as of 2006 [1]. The common factors that contribute to the development of MetS include obesity, diabetes, hypertension, and hyperglycemia. MetS develops as a result of an imbalance among dietary intake, sedentary lifestyle, glucose metabolism and cardiac control [2]. Interestingly, all of these factors also play a crucial role in proper functioning of the kidneys. Oxidative stress triggered by the overproduction of reactive oxygen species (ROS) or inefficient antioxidant systems is also involved in the development of renal injury [3]. A wide range of pharmacological therapeutics is available to combat the factors that lead to MetS, but most have side effects. In this context, the need for non-pharmacological approaches to delay the progression of MetS is growing. Many fruits, especially berries, have been shown to be excellent sources of antioxidant compounds, such as anthocyanins and phenolics [3].

Blueberries (BB), (*Vaccinium sp.*) have the highest antioxidant capacity among fruits, supplemented by anthocyanins, proanthocyanins, flavanols and phenolic acid present in them [4]. BB possess known anti-inflammatory and antioxidant properties. We have previously shown that

hypertensive rats have increased production of proinflammatory cytokines (PIC) and renin-angiotensin system (RAS) components [3], and when fed with a BB diet, exhibit reduced oxidative stress and improved nephropathy [5]. We recently established that BB protect against LPS-induced acute kidney injury by modulating TLR4 expression [6]. Although previous studies have reported the beneficial effects of BB, the mechanism by which BB antioxidants protect against MetS-induced renal injury has not been explored.

Inflammation triggered by excessive PIC production is associated with hypertension-induced renal injury and cardiac pathology [7]. PIC have been shown to exacerbate ROS generation [8, 9], which can activate several intracellular signaling pathways including the NF κ B pathway [3]. The NF κ B signaling pathway leads to the production of more PIC, which in turn increases ROS generation and therefore gives rise to a vicious cycle. In MetS, at the cellular level, when a cell is under stress from increased fat or sugar concentration, the cell surface receptors detect the changes in the external environment, trigger the production of PIC, and thereby generate ROS. Hence, the cell surface receptors play an important role in initiating inflammation in MetS.

Toll-like receptors (TLRs) are pattern recognition receptors that play key roles in the innate immune system. TLRs detect pathogens or danger-associated molecular patterns and initiate immune cell responses. Toll-like receptor 4 (TLR4), in particular, is shown to induce cytokine production and expression of co-stimulatory molecules [10] via the NF κ B signaling pathway [11, 12]. TLR4 expression is increased in human macrophages in lipid-rich plaques [13], indicating TLR4 activation by hyperlipidemia. Further, TLR4, along with TLR2, has been shown to be increased in adolescents with MetS [14].

Mitogen-activated protein kinases (MAPKs) are important mediators of inflammation-induced tissue injury. Interestingly, MAPKs are critical downstream regulators of TLR pathways [15, 16]. Although MAPK activation has been studied in inflammation-associated organ dysfunction [17, 18] and has been shown to contribute to insulin resistance [19], the involvement of TLR4-mediated MAPK phosphorylation in MetS-induced kidney dysfunction has been poorly understood. The objective of this study was to assess the role of TLR4 in MetS-induced renal damage and to understand the mechanisms by which BB protect against CKD. We hypothesized that the renal damage induced by MetS is caused by TLR4 activation and that BB protect against the damage by inhibiting TLR4 expression and subsequent downstream MAPK phosphorylation.

4.2 MATERIALS AND METHODS

Ethics Statement

All experimental procedures were in compliance with all applicable principles set forth in the National Institutes of Health 2011 Guide for the Care and Use of Laboratory Animals. This study was approved by the Institutional Animal Care and Use Committee of the Louisiana State University School of Veterinary Medicine (protocol approval number 09-008).

Animals

Five-week old heterozygous LZR (*fa/+*) and homozygous OZR (*fa/fa*) were purchased from Harlan and housed in a temperature- ($23\pm 2^{\circ}\text{C}$), humidity- and light- (12 hour light/dark cycle) controlled environment. The baseline body weights of the rats ranged from 130 to 150 grams.

Experimental design

Rats were randomly divided into four different groups: LZR Control (LZRCC), LZR Blueberry (LZRBB), OZR Control (OZRCC), and OZR Blueberry (OZRBB). Animals were fed control or BB-enriched diets for 15 weeks. All animals were subjected to acute determination of glomerular filtration rate (GFR) and renal plasma flow (RPF) at the end of the 15 week feeding period. Rats were euthanized and kidneys were excised for analyses. Kidneys were formalin-fixed, paraffin-embedded, and then sectioned (3 μ m).

Diets

Diets were prepared by Harlan Teklad (Madison, WI) using a reformulated NIH-31 diet by adding 20g/kg lyophilized BB or 20g/kg dried corn. The 2% BB diet was prepared by homogenizing the berries in water, lyophilizing and adding the preparation to the NIH-31 rodent chow. The control diet was prepared with corn instead of BB and the amount of corn in the control diet was adjusted to compensate for the added volume of BB, in order to make the two diets isocaloric [5]. The food consumption was measured weekly for all four groups.

Glucose tolerance test

Blood was obtained by tail nick in conscious, restrained animals. Glucose was measured with a handheld glucose meter (One Touch Ultra, USA). Baseline fasting blood glucose was determined after an overnight fast. A glucose bolus (2g/kg of body weight) was then administered intraperitoneally, and blood glucose was measured at 15, 30, 45, 60, 90, 120, 150, and 180-minute intervals.

Acute renal clearance experiments

The OZR animals have been shown to exhibit reduced renal function parameters, namely glomerular filtration rate (GFR) and renal blood flow (RBF) even at 12-14 weeks of age. [20, 21]

The average GFR of male obese zucker rats at 20 weeks of age was 0.3ml/min/g kidney wt.[21] and RBF at 14 weeks was shown to be 6ml/min/g kidney wt. Rats from all the study groups were subjected to renal clearance experiments at the end of the feeding period [3, 6]. Briefly, each rat was anesthetized with inactin (thiobutabarbital; 100mg/kg body weight), the right inguinal area was shaved, a small incision made, and femoral vessels were carefully isolated. The femoral artery was cannulated with heparin-primed (100U/ml) PE-50 polyethylene tubing connected to a pressure transducer (PowerLab data acquisition systems; AD Instruments, Colorado Springs, CO) for continuous measurement of arterial pressure. The femoral vein was catheterized with heparin-primed PE-50 tubing for infusion of solutions at 20 μ l/min. An isotonic saline solution containing 6% albumin was infused during surgery. After surgery, the infusion solution was replaced with isotonic saline containing 2% bovine serum albumin (BSA), 7.5% inulin (Inutest) and 1.5% PAH, and a 300 μ l bolus of this solution was administered at the start of each clearance experiment. The bladder was exposed via a suprapubic incision and catheterized with a PE-200 tube for gravimetric urine collection. After a 15-20 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 as previously described ([5, 6]. Plasma inulin and PAH concentrations were measured colorimetrically to determine GFR and RPF, respectively.

Glomerular injury scoring

A semi-quantitative glomerular scoring method was used, based upon previously published methods for glomerular scoring [5]. The method was expanded to include parietal metaplasia of Bowman's capsule, glomerular sclerosis, proteinuria and interstitial nephritis.

Measurement of Urinary Albumin levels

Urine albumin was assessed in animals from all experimental groups using the NephraII Albumin Assay Kit (Exocell Inc., Philadelphia, USA) according to manufacturer's instructions.

Electron paramagnetic resonance (EPR) spectroscopy

Total ROS, superoxide, and peroxynitrite production rates were measured in pieces of kidney cortex via EPR spectroscopy as previously described [5, 6, 22-25]. The term 'total ROS' represents all ROS; 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°C with 6.6µl of CMH (200µM) for total ROS measurement; 1.5µl of PEG-SOD (50U/µl) for 30 minutes, then CMH for an additional 30 minutes for superoxide measurement; or 30µl of CPH (500µM) for 30 minutes for peroxynitrite measurement. Aliquots of incubated probe media were taken in 50µl disposable glass capillary tubes (Noxygen Science Transfer and Diagnostics, Elzach, Germany) for determination of total ROS, superoxide or peroxynitrite production, under previously established EPR settings [3, 5, 6].

RNA extraction and Real-Time PCR

Real-time PCR was used to determine the expression levels of kidney cortex (KC) PIC and TLR4. Total RNA was isolated using Trizol reagent (Invitrogen, CA). The RNA concentration was calculated from the absorbance at 260nm and RNA quality was assured by the 260/280 ratio. The RNA samples were treated with DNase I (Ambion) to remove any genomic DNA. First strand cDNA were synthesized from 2µg RNA with iScript cDNA synthesis kit (Bio-rad, Hercules, CA). Real-time PCR was performed in 384-well PCR plates using iTaq SYBR Green Super mix with ROX (Bio-rad) in triplicate using the ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA). The PCR cycling conditions were as follows:

50°C for 2 mins, 95°C for 3 min, followed for 45 cycles (15s at 95°C and 1 min at 60°C). To confirm the specific PCR product, a dissociation step (15s at 95°C, 15s at 60°C and 15s at 95°C) was added to check the melting temperature. Gene expression was measured by the $\Delta\Delta CT$ method and was normalized to 18s RNA mRNA levels. The data presented are the fold changes of the gene of interest relative to that of the control animals.

Western Blotting analysis

Primary rat TLR4 (sc-30002), IL-1 β (sc-7884), IL-18 (sc-7954), Nrf2 (sc-722) and Keap1 (sc-33569) antibodies were purchased from Santa Cruz Biotechnology, USA and p38MAPK (#9212), p-p38MAPK (#9211), ERK1/2 (#9102) and p-ERK1/2 (#9106) from Cell Signaling, USA and used following manufacturer-recommended dilutions, followed by a horseradish peroxidase-conjugated anti-rabbit IgG antibody (SC-2004, Santa Cruz Biotechnology, Inc). Rat primary anti-GAPDH (sc-25778) or anti-actin (sc-1616) was used to confirm the loading and the transfer. We used ImageJ software to analyze the bands.

Measurement of cortical catalase and superoxide dismutase levels

Antioxidant levels in renal cortex of animals from all experimental groups were quantified. Catalase levels were assessed with a catalase assay kit (Cayman Chemicals, Ann Harbor, MI) and superoxide dismutase enzyme levels were determined using a SOD assay kit (Dojindo Molecular Technologies, Rockville, Maryland) according to manufacturer's instructions.

Quantification of NF κ B p65 activity

The NF κ B/p65 activity ELISA (Active Motif, USA) kit was used to assess the binding activity of free NF κ B p65 in nuclear extracts, as described previously [6]. A sandwich ELISA method was employed to perform the analysis, according to the manufacturer's instructions.

4.3 RESULTS

Body weight and food intake

Food consumption and body weight were measured weekly. Food intake did not differ between LZRCC and LZRBB animals or between OZRCC and OZRBB groups. The initial and final body weights of the animals in all groups are summarized in Table 1. OZRCC and OZRBB had significantly higher body weights compared to the LZR animals that were fed either diet. There was no significant difference between the body weights of OZR rats, irrespective of the diet.

BB-enriched diet improves glucose tolerance in MetS animals

The control OZR had impaired glucose tolerance compared to the LZR. The improvements in glucose tolerance over different time-points in BB-fed OZR rats appear in Figure 4.1. BB feeding for a period of 15 weeks improved glucose tolerance in OZRBB rats compared to the OZRCC group. However, there was no significant difference in the glucose sensitivity between LZRCC and LZRBB rats.

Table 4.1: Average initial and final body weight of rats. The body weight of all rats was measured at the start and end of the feeding period. All values are presented as mean \pm SEM (*- $p < 0.05$ vs. LZRCC, # - $p < 0.05$ vs. OZRCC, \$- $p < 0.05$ vs. OZRBB)

Body Weight	LZR		OZR	
	CC	BB	CC	BB
Initial weight (g)	158.4 \pm 3.99	163.9 \pm 2.96	184.2 \pm 3.56*	181.6 \pm 5.14*
Final weight (g)	554 \pm 12.07	531.7 \pm 15.26	840.1 \pm 42.64*	814 \pm 46.24*

Effect of BB feeding on glucose sensitivity in MetS animals

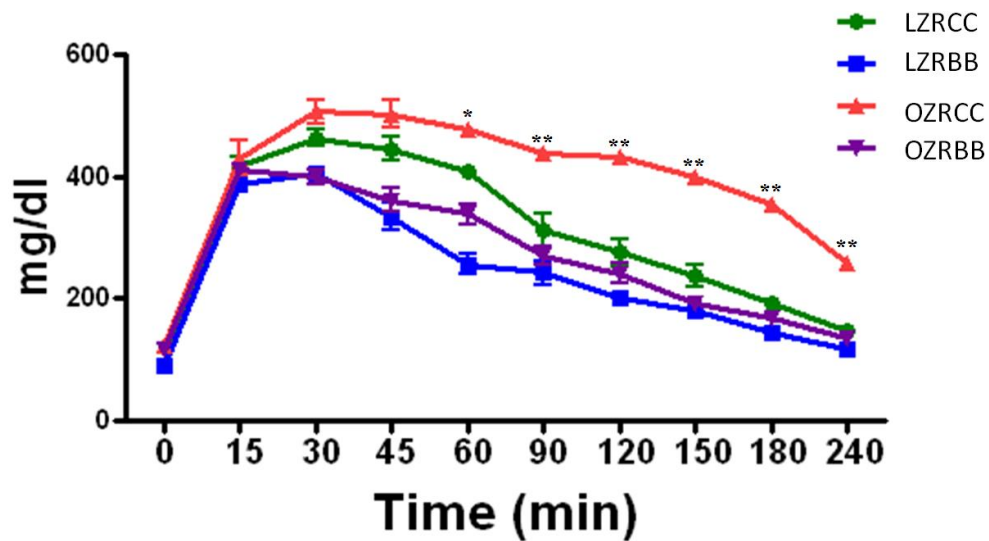


Figure 4.1: Effect of BB feeding on glucose sensitivity in MetS animals. The glucose sensitivity of rats from all experimental groups as assessed by glucose sensitivity assay (n=7 per group). All values are presented as mean \pm SEM (*p < 0.05, **p < 0.01)

Kidney function is impaired in OZR and blueberry improves renal hemodynamic parameters in these animals

MetS is known to induce a loss of function of the kidney, thus leading to alterations in renal hemodynamic parameters. The kidney function in rats from all study groups was assessed to determine the extent of kidney damage. The blood pressure trends for each group of rats appear in Figure 4.2A. The OZRCC rats had significantly higher mean arterial pressure (MAP) compared to the LZR rats. The MAP of OZRBB rats were significantly lower in comparison to the OZRCC group by the end of the feeding period. Figures 4.2B-4.2D illustrates the reno-protective effects of BB-enriched diet in OZR rats fed for 15 weeks. The renal clearance experiments showed significant reductions in GFR and RBF, and an increase in renal vascular resistance (RVR) in OZRCC animals. In contrast, in the OZRBB rats, there was a significant increase in GFR and RBF, and a decrease in RVR, indicating a reno-protective effect of BB. There was no significant difference in blood pressure or renal hemodynamic measures between LZRCC and LZRBB animals.

In addition to the kidney function parameters, we also measured the expression of renin-angiotensin system (RAS) genes in the kidneys of animals from all experimental groups (Figure 4.3A-4.3D). The OZRCC animals had an elevated expression of ACE and AT1 genes and a significantly reduced expression of ACE2 and AT2, compared to the lean controls. Conversely, BB enriched diet could significantly attenuate the expression of ACE and AT1, and also improved the expression levels of ACE2 and AT2 genes in the renal tissue of OZR rats. Taken

Effect of BB feeding on renal function in MetS animals

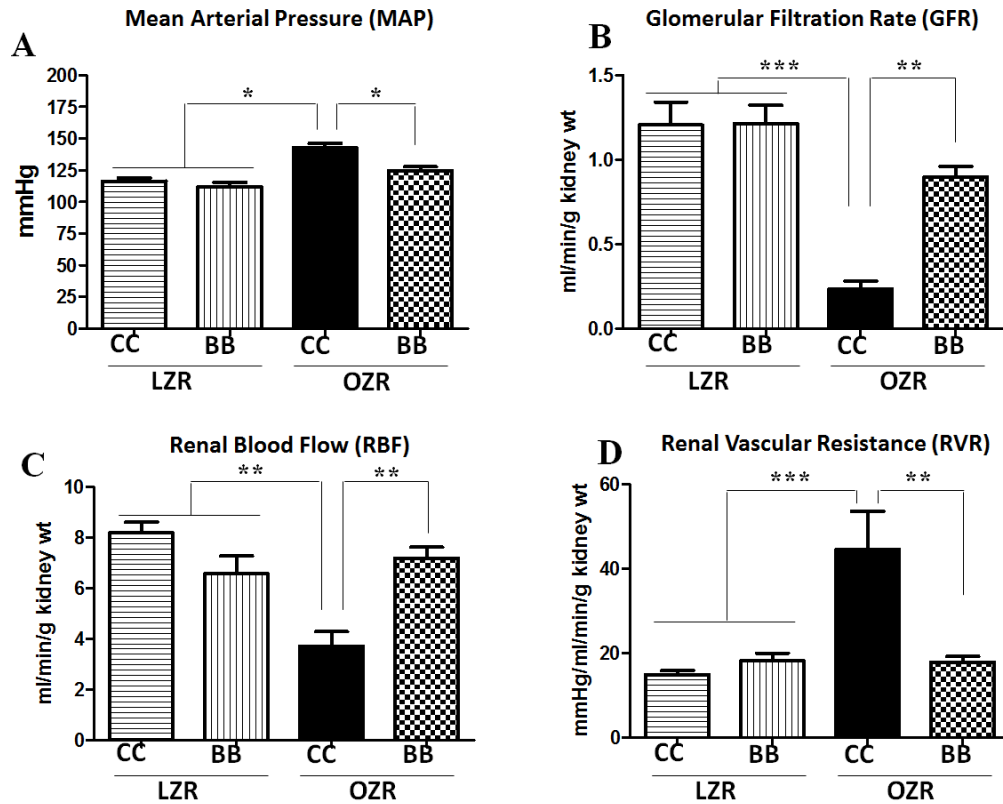


Figure 4.2: Effect of BB on renal hemodynamic dysfunctions in MetS animals. The kidney function of rats from all experimental groups as assessed by renal clearance experiments (n=6-9 per group) (A) BB supplementation significantly decreased the MAP in obese Zucker rats (B) MetS animals exhibited a significantly reduced GFR. BB pretreatment improved the GFR in MetS animals. (C) MetS animals had a reduced renal blood flow. BB treatment normalized the renal blood flow in these animals. (D) The RVR was increased in MetS animals and BB prevented this increase in RVR in them. All values are presented as mean \pm SEM (* p < 0.05, ** p < 0.01, *** p < 0.001)

Effect of BB feeding on the renal renin-angiotensin system in MetS animals

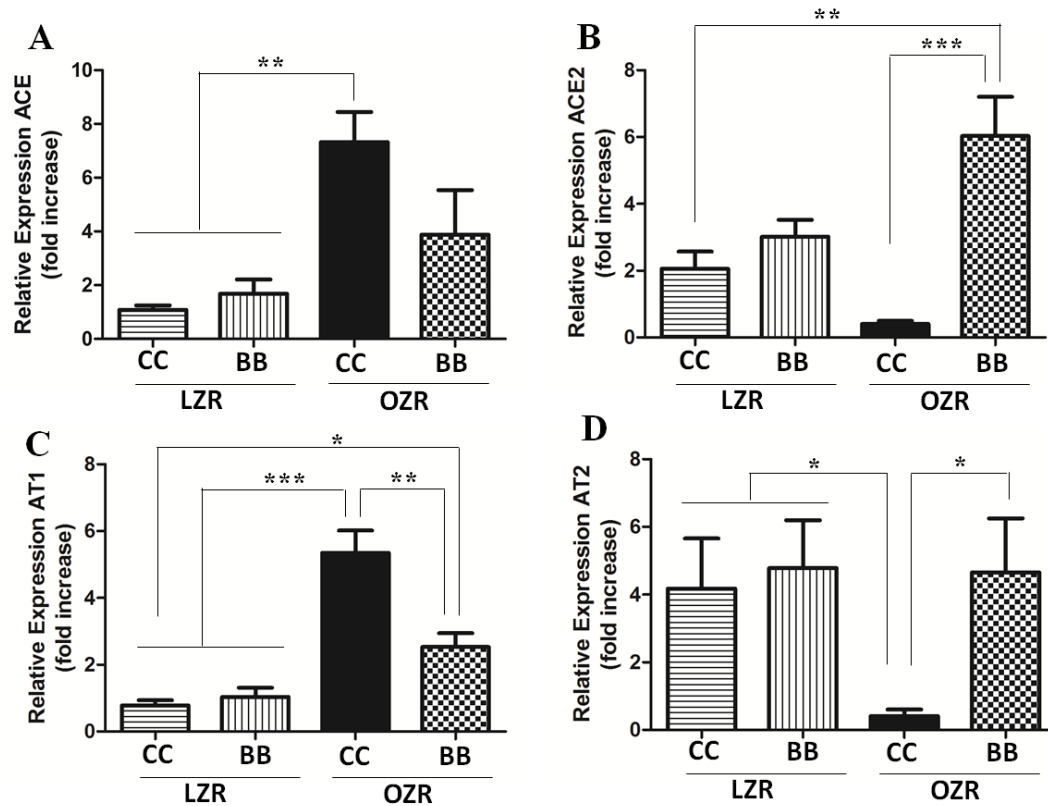


Figure 4.3: Effect of BB on renal renin-angiotensin system in MetS animals. The mRNA expression of (A) ACE, (B) ACE2, (C) AT1 and (D) AT2 in the renal cortical tissue. The gene expression of ACE and AT1 were significantly increased and ACE2 and AT2 were reduced in MetS rats. The BB enriched diet was able to attenuate these changes in renin-angiotensin system gene expression. All values are presented as mean \pm SEM (* p < 0.05, ** p < 0.01, *** p < 0.001)

together with the improved renal function, this result of the renin-angiotensin system components confirms a protective functional role of BB in the kidneys of MetS animals.

Kidney structure is altered in OZR and blueberry feeding preserves glomerular morphology and structure in these animals

Masson's Trichrome-stained kidney sections from rats (n = 6) from all experimental groups were examined by a veterinary pathologist who was blinded to the experimental conditions. The kidneys of LZRCC and LZRBB exhibited similar appearances histologically. Representative photographs of glomeruli for each experimental group appear in Figure 4.4. Renal changes in the OZRCC image are evident in glomeruli, tubules and interstitium. There is moderate glomerular fibrosis; tubular dilatation and attenuation of lining epithelium with intraluminal hyaline casts; tubular atrophy; multifocal to coalescing moderate to severe interstitial fibrosis; multifocal interstitial, moderate to severe lymphocytic and plasmacytic nephritis. Remarkably, OZRBB animals exhibited less pathological changes with minimal interstitial fibrosis (Figure 4.5). When compared to OZRCC, OZRBB exhibited less severe glomerular adhesions, cortical and medullary tubular lesions, interstitial nephritis.

Oxidative stress contributes to the progression of MetS while blueberry diet attenuates free radical production rates in MetS animals

Overproduction of free radicals, mainly ROS and reactive nitrogen species (RNS), is a major factor contributing to the progression of MetS. We measured total ROS, superoxide and peroxynitrite production rates using EPR spectroscopy in cortical tissues from rats (n=7) in all experimental groups (Table 4.2). At the kidney tissue level, the total ROS, superoxide and peroxynitrite production rates were significantly higher in OZRCC rats compared to the LZRCC and LZRBB rats. However, BB treatment attenuated the rate of cortical and medullary free

radical production in the OZRBB animals. Table 4.2 shows the free radical production rates in cortical and medullary kidney tissues in rats from all experimental groups.

Effect of BB feeding on the renal pathology in MetS animals

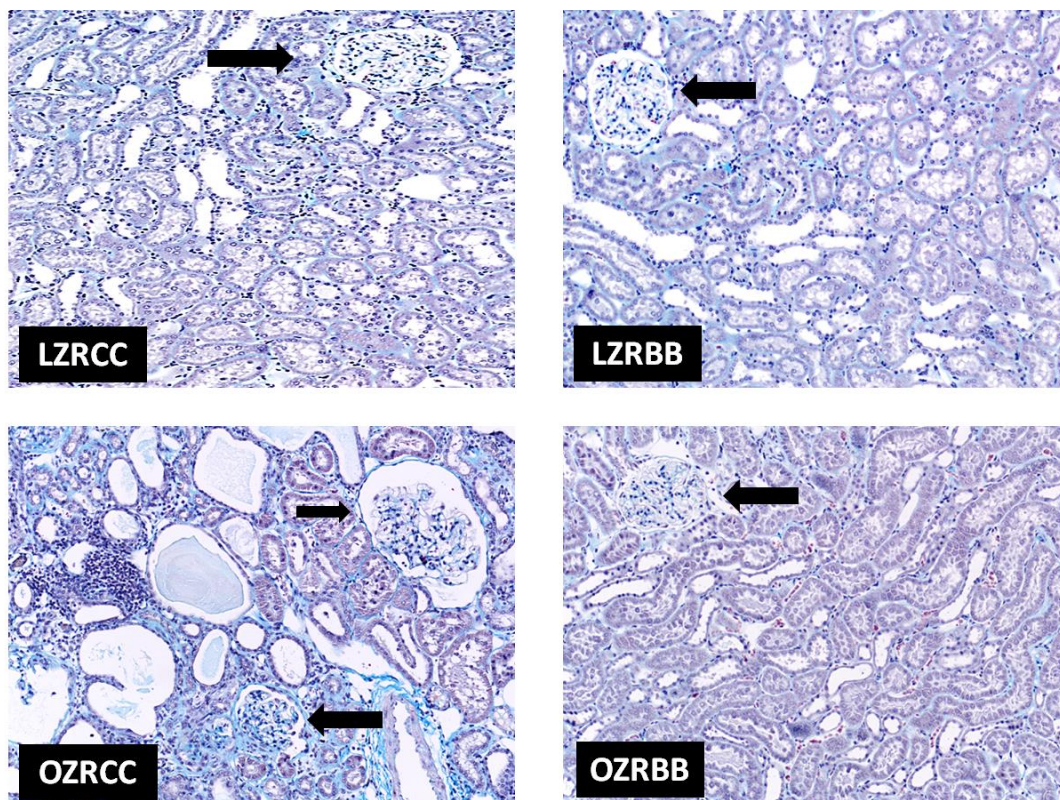


Figure 4.4: Effect of BB on renal pathology in MetS animals. (A) Representative images of the renal pathology of rats from all experimental groups as assessed by Masson's trichrome staining of the kidney sections (n=6). There are no significant renal structural changes visible in LZRCC and LZRBB groups. However, renal changes in the OZRCC image are evident in glomeruli, tubules and interstitium. There is moderate glomerular fibrosis; tubular dilatation and attenuation of lining epithelium with intraluminal hyaline casts; tubular atrophy; multifocal to coalescing moderate to severe interstitial fibrosis; multifocal interstitial, moderate to severe lymphocytic and plasmacytic nephritis. OZRBB animals exhibited less pathological changes with minimal interstitial fibrosis.

Effect of BB feeding on the renal pathology in MetS animals (pathological scoring)

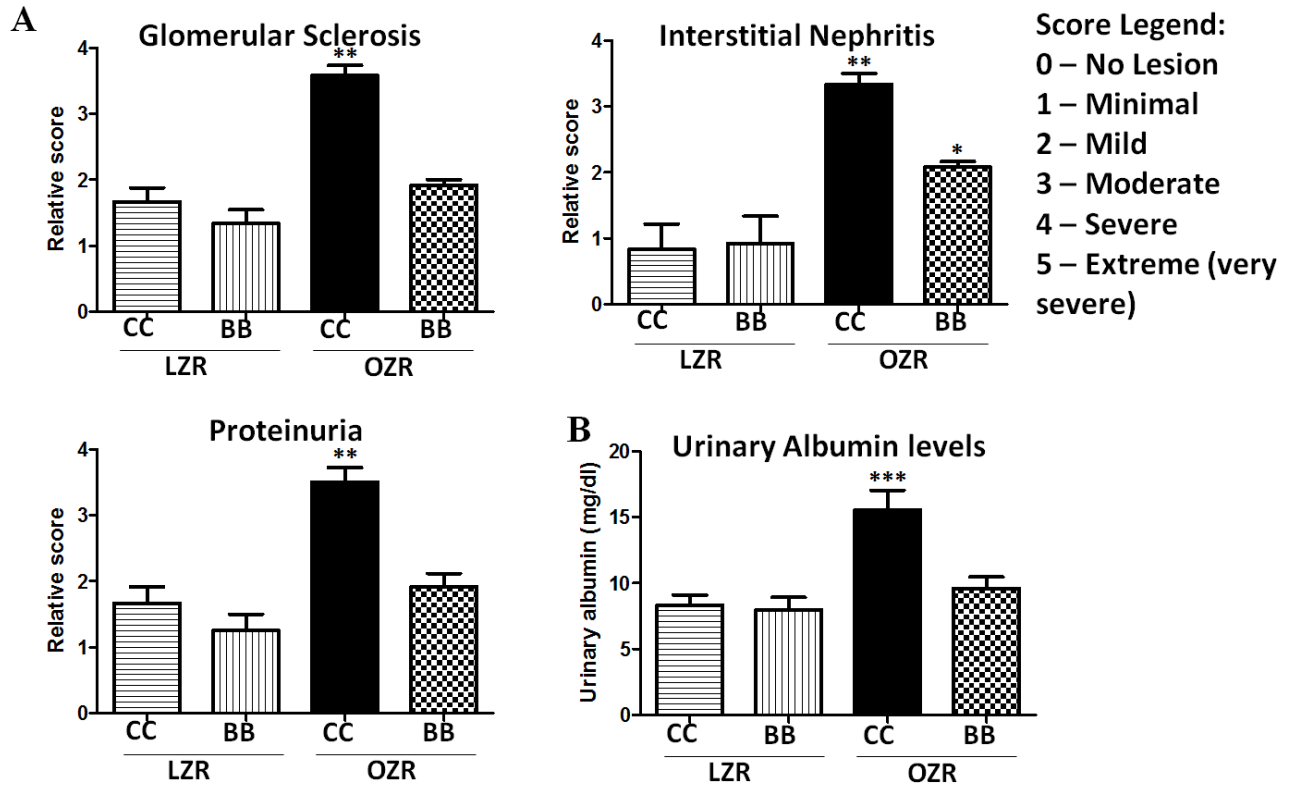


Figure 4.5: Effect of BB on renal pathology in MetS animals (pathological scoring). (A) Relative pathological scoring of glomerular sclerosis, interstitial nephritis and proteinuria as evaluated by a veterinary pathologist who was blinded to experimental conditions (n=6). (B) Urinary albumin levels (mg/dl) in rats from all experimental groups as determined by the albumin assay kit. (** $p < 0.01$ to LZRCC, *** $p < 0.001$ to LZRCC)

Table 4.2: Rate of generation of total ROS, superoxide and peroxynitrite in the kidney cortical and medullary tissue. OZRCC animals showed a significantly higher rate of total ROS, superoxide and peroxynitrite generation. Pretreatment with BB was able to inhibit the increase in the rate of free radical production. All values are presented as mean \pm SEM (*- p < 0.05 vs. LZRCC, # - p < 0.05 vs. OZRCC, \$- p < 0.05 vs. OZRBB)

	LZR		OZR	
	CC	BB	CC	BB
Renal Cortex				
Total ROS (μ M/mg protein/min)	0.138\pm0.026	0.131\pm0.011	0.368\pm0.055^{*\$}	0.224\pm0.026^{*#}
Superoxide (μ M/mg protein/min)	0.048\pm0.009	0.037\pm0.012	0.089\pm0.021^{*\$}	0.061\pm0.014^{*#}
Peroxynitrite (μ M/mg protein/min)	0.017\pm0.005	0.020\pm0.003	0.072\pm0.013^{*\$}	0.033\pm0.015^{*#}
Renal Medulla				
Total ROS (μ M/mg protein/min)	0.074\pm0.012	0.067\pm0.015[*]	0.182\pm0.020^{*\$}	0.092\pm0.016^{*#}
Superoxide (μ M/mg protein/min)	0.027\pm0.008	0.024\pm0.009	0.098\pm0.015^{*\$}	0.053\pm0.008^{*#}
Peroxynitrite (μ M/mg protein/min)	0.011\pm0.006	0.010\pm0.006	0.036\pm0.007^{*\$}	0.016\pm0.010[#]

Kidney TLR4 expression is elevated in animals with MetS and blueberry supplementation decreases TLR4 expression in the kidney

TLR4 plays a vital role in activating the immune system in response to inflammation and oxidative stress. Kidney cortical TLR4 gene and protein expression was measured in tissues from all experimental groups using real time RT-PCR and western blotting technique respectively (Figure 4.6). Both gene and protein levels of TLR4 were elevated in OZRCC rats compared to the LZR animals, indicating that the increased inflammation in the kidney of MetS animals was, at least in part, mediated through TLR4. In contrast, OZRBB rats had an attenuated TLR4 gene and protein expression levels when compared to OZRCC rats, and comparable to LZRCC and LZRBB animals.

MetS animals expressed increased phosphorylation of ERK and p38 MAPK and BB intervention significantly inhibited the phosphorylation of MAPK

We examined the expression levels of total and phosphorylated ERK and p38 in the renal cortex of animals from all experimental groups. The expression level of p-ERK and p-p38MAPK was not significantly different between the LZR animals (Figure 4.7A-4.7C). Consistent with the TLR4 expression pattern, the OZRCC exhibited an elevated expression of p-ERK and p-p38MAPK in the kidneys. In contrast, BB intervention (OZRBB) attenuated the phosphorylation of MAPK in these animals. Taken together with the reduced TLR4 expression in MetS rats, these findings clearly demonstrate that BB pretreatment in MetS animals attenuates MAPK expression.

Effect of BB feeding on renal TLR4 gene and protein expression in MetS animals

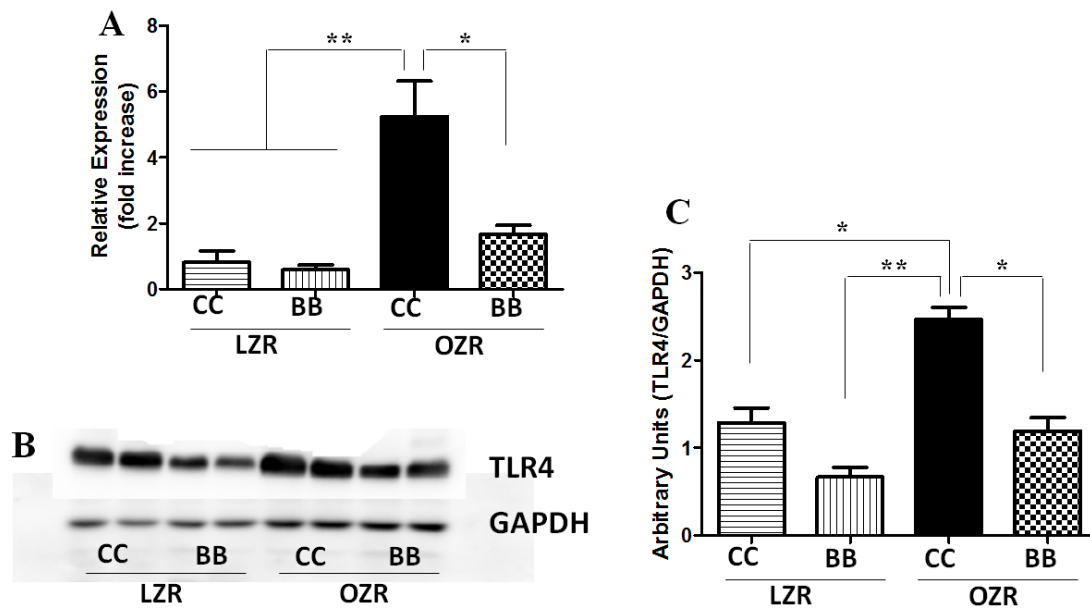


Figure 4.6: Effect of BB on renal TLR4 gene and protein expression in MetS animals. The (A) mRNA (n=6) and (B) a representative western blot of TLR4 in the renal cortical tissues. (C) Bands were analyzed and quantified by densitometry (n=6). The gene and protein expression of TLR4 was increased in the MetS rats. The pretreatment with BB was able to inhibit the increased expression of TLR4 in MetS animals and was comparable to the LZR controls. All values are presented as mean \pm SEM (*p < 0.05, **p < 0.01)

Effect of BB feeding on ERK and p38MAPK expression in MetS animals

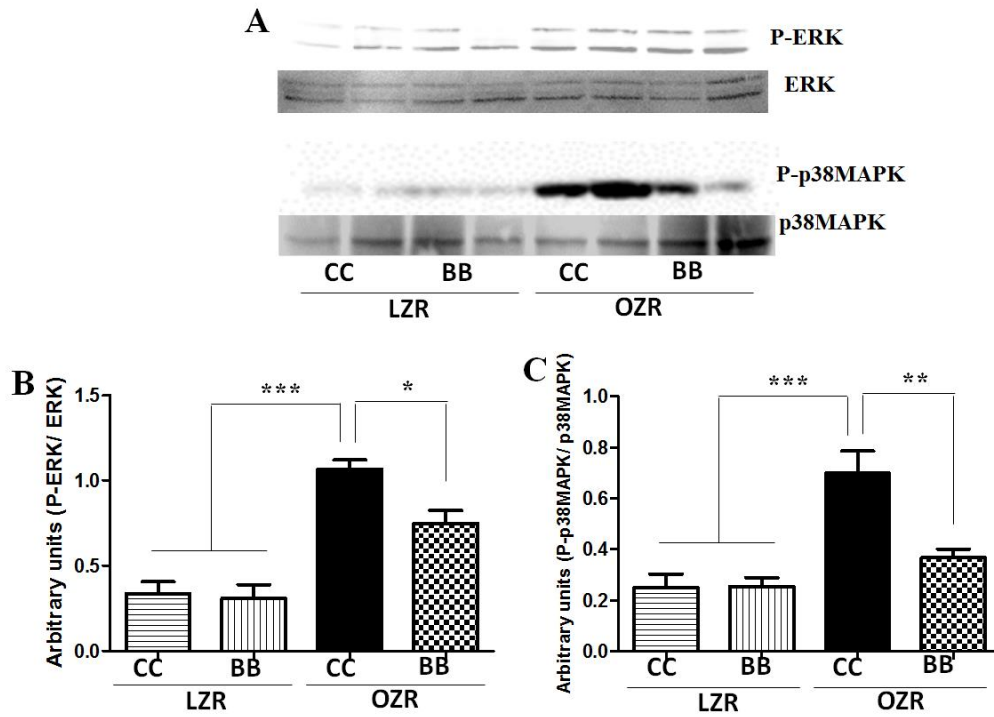


Figure 4.7: Effect of BB on MAPK signaling in MetS animals. The protein expression of total and phosphorylated MAPK (ERK and p38) was determined by western blotting. (A) A representative western blot showing ERK and p38MAPK protein expression. Western bands for ERK (B) and p38MAPK (C) were analyzed and quantified by densitometry. All values are presented as mean \pm SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

NFκB activity and PIC expressions are higher in MetS and blueberry feeding attenuates these changes in MetS animals

The activation of TLR4 was confirmed by measuring the NFκB activity. We measured the renal cortical NFκB p65 DNA binding activity in tissues from control and BB treated groups (n=6). Consistent with the TLR4 expression results, rats from OZRCC group had significantly higher cortical NFκB activity compared to LZRCC and LZRBB groups (Fig 4.8B). The OZRBB rats had a significantly lower NFκB activity levels compared to the OZRCC rats suggesting that the BB supplementation prevents the increase in NFκB activity seen in MetS animals.

Gene expression levels of proinflammatory cytokines IL-1β, IL-18, TNFα and protein expression levels of IL-1β and IL-18 were measured in all experimental groups (n=6). These interleukin levels were significantly reduced in the OZRBB compared to the OZRCC animals (Figure 4.8A, 4.8C-4.8E). Again, there was no significant difference in the expression of these proteins between the two groups of LZR animals. The difference in the expression of IL-1β and IL-18 between the OZR groups is particularly important not only because these are proinflammatory molecules, but also as IL-1β and IL-18 are downstream molecules of the TLR4 pathway. TNFα mRNA expression results were also consistent with the expression of other PIC.

MetS animals had a reduction in antioxidant defense which was restored by the blueberry diet

We measured the cortical levels of the antioxidant enzymes SOD and catalase in all experimental groups (Figure 4.9A-4.9B). SOD catalyzes the conversion of superoxide into oxygen and hydrogen peroxide. Catalase breaks down hydrogen peroxide. The SOD and catalase levels were depleted in rats belonging to the OZRCC group compared to the LZR groups. A blueberry-enriched diet significantly increased the SOD and catalase levels in the OZRBB group,

indicating a beneficial antioxidant effect of BB in MetS animals. We also determined the protein expression levels of redox sensitive transcription factor NF-E2-related factor 2 (Nrf2), which is a

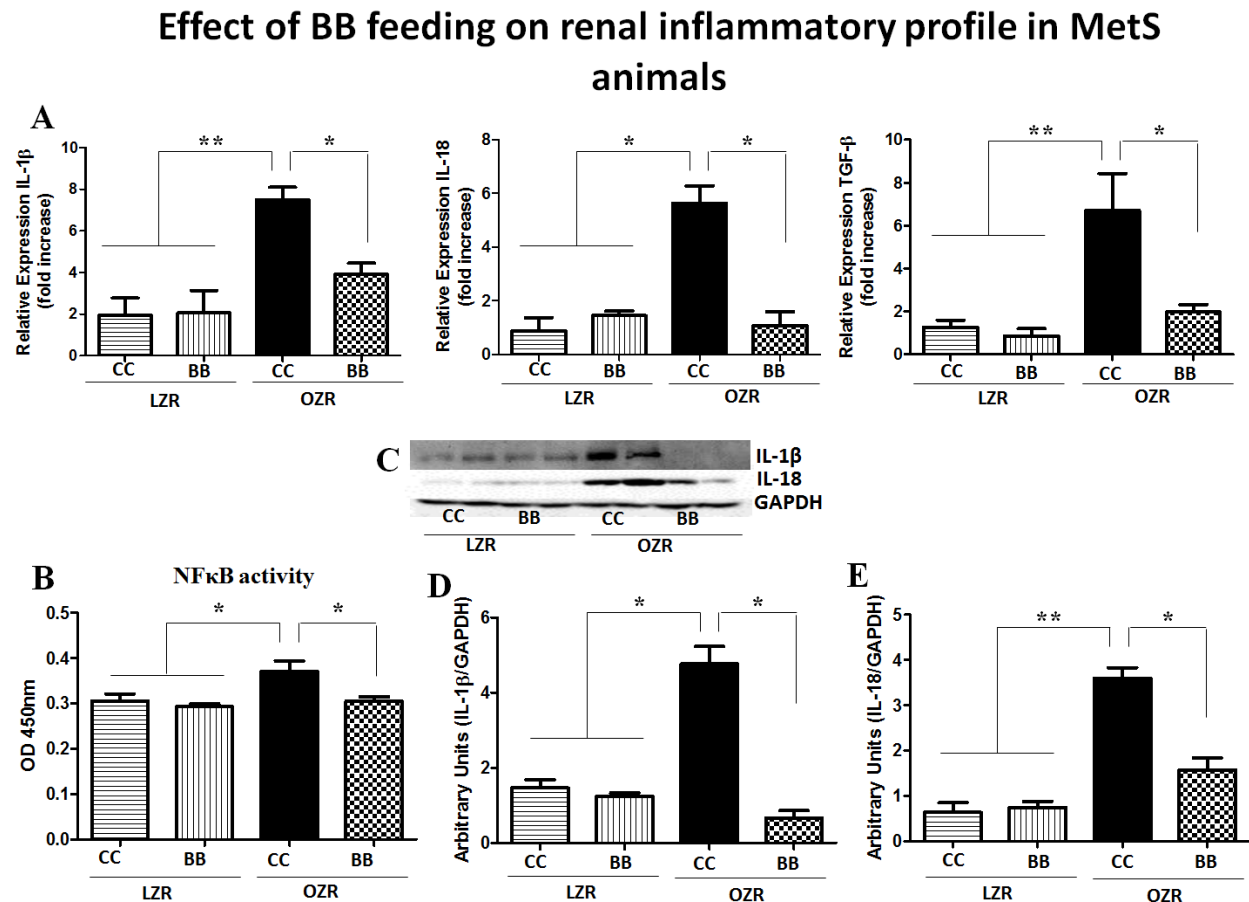


Figure 4.8: Effect of BB on the renal inflammatory profile in MetS animals. The (A) mRNA expression (n=6) of IL-1 β , IL-18 and TGF- β in the renal cortical tissues. (B) DNA binding activity of NF κ B p65 subunit in renal cortical tissues of rats from each experimental group (n=6), as determined by ELISA. Increased NF κ B p65 DNA binding activity in MetS animals was significantly reduced by BB treatment. (C) A representative western blot showing IL-1 β and IL-18 protein expression. Western blot bands for IL-1 β (D) and IL-18 (E) were analyzed and quantified by densitometry. All values are presented as mean \pm SEM (*p < 0.05, **p < 0.01)

Effect of BB feeding on antioxidant enzymes and Nrf2-Keap1 signaling in MetS animals

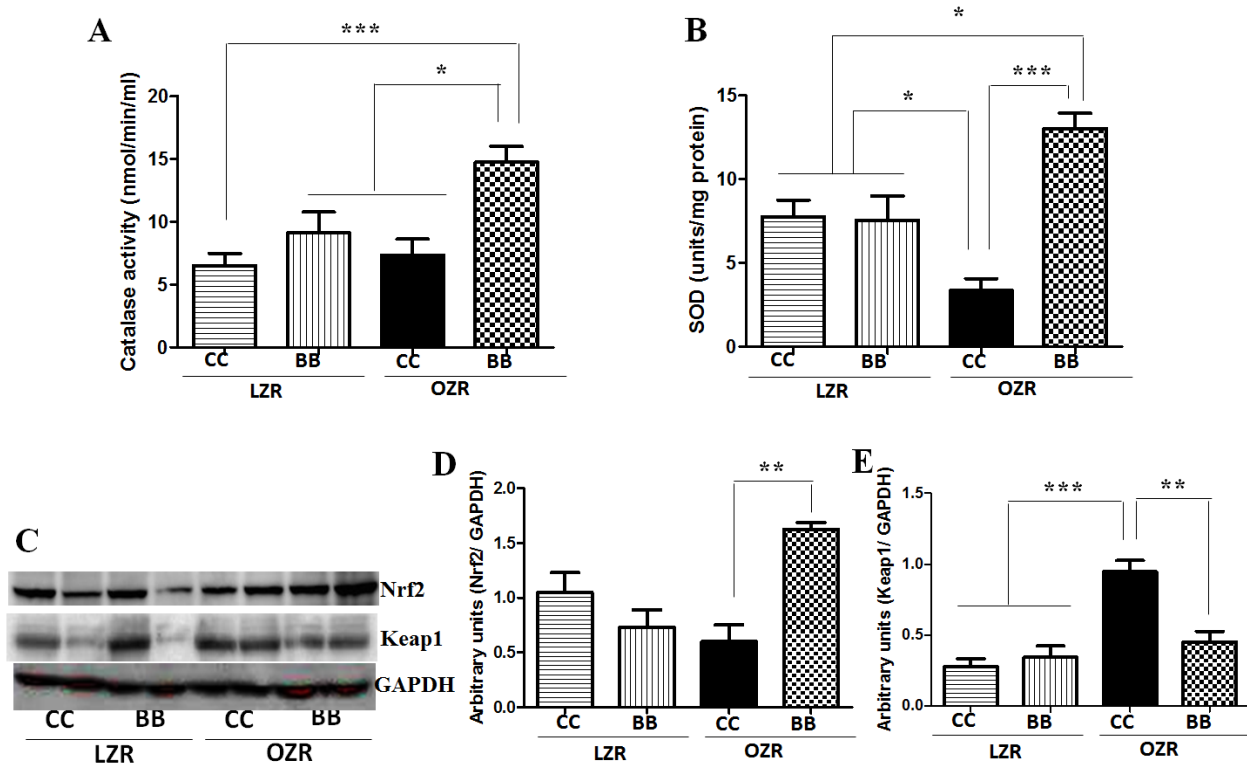


Figure 4.9: Effects of BB on antioxidant enzymes and Nrf2-Keap1 signaling in MetS animals. Renal cortical catalase (A) and SOD (B) levels as determined by commercially available kits (n=6). (C) A representative western blot showing Nrf2 and Keap 1 protein expression. Western bands for Nrf2 (D) and Keap1 (E) were analyzed and quantified by densitometry. All values are presented as mean \pm SEM (*p < 0.05, **p < 0.01, ***p < 0.001)

key regulator of antioxidant enzymes and its associated protein Kelch-like ECH associated protein 1 (Keap1) in the kidney of animals from all the groups. Nrf2 levels were significantly increased and Keap1 concomitantly reduced in the OZRBB group compared to the also determined the protein expression levels of redox sensitive transcription factor NF-E2-related factor 2 OZRCC (Figure 4.9C-4.9E), indicating an improved anti-oxidant defense system in BB fed MetS animals.

4.4 DISCUSSION

The most prominent finding of this investigation is that BB protect against chronic kidney disease in the zucker rat model of metabolic syndrome by attenuating TLR4 expression and reducing oxidative stress in the kidney. To the best of our knowledge, this study is the first to demonstrate that 1) TLR4 signaling and consequent inflammatory cascade, at least in part, mediates the renal dysfunction in MetS animals; 2) TLR4-mediated phosphorylation of ERK and p38MAPK could be a possible mechanism for the progression of chronic kidney injury in MetS; 3) BB can protect against chronic kidney injury in MetS animals by inhibiting TLR4 and subsequently attenuating MAPK activity; and 4) BB can improve other contributing factors to MetS, such as glucose metabolism, by reducing inflammation and improving the redox balance. The ability of BB to inhibit TLR4 could be an important advancement towards the identification of novel therapeutic strategies.

MetS is a progressive health disorder that predisposes an individual to cardio-renal dysfunction [26] and its increasing incidence is a matter of concern. The most effective way to prevent kidney disease induced by MetS is to prevent the incidence of MetS or to delay its progression. However, the mechanism by which MetS exacerbates kidney disease remains elusive. In this study, we used zucker rats which are established genetic models of research for

MetS. The obese Zucker rats (recessive) are characterized by obesity, hyperinsulinemia, hypertension and hyperlipidemia. Interestingly, inflammation and oxidative stress have been implicated in all these pathological conditions and also in CKD [27]. Therefore, we sought to investigate the mechanism that regulates the CKD in these animal models of MetS. We also studied if a preventive intervention by BB could attenuate the structural and functional damage to the kidney in these MetS animals. Further, we tried to elucidate a molecular mechanism by which BB protects against MetS-induced kidney dysfunction.

Chronic kidney disease is characterized by a loss of function of the kidneys, which disrupts the body fluid homeostasis. Recent studies have demonstrated that kidney disorders are impending in MetS [27] and TLR4 blockade protects the kidney in type-2 diabetic mice via modulating the inflammatory signaling [28]. In this study, we analyzed renal functional parameters in all experimental groups. Our findings indicate that the OZRCC rats had a marked decrease in kidney function as evidenced by a reduced GFR and RBF, and increased RVR when compared to the lean controls. These findings are consistent with previous studies demonstrating that MetS and its components are associated with a reduction in GFR [29, 30]. However, renal functional parameters were normalized in the blueberry fed OZR (OZRBB) rats, demonstrating a significant increase in the GFR and RBF, and decrease in the RVR compared to their control diet-fed obese counterparts.

Abnormal activation of the RAS is an important mediator of kidney damage [31] and has proinflammatory effects that in turn affect the renal hemodynamic parameters [32]. We measured the gene expression levels of RAS components (ACE, ACE2, AT1, AT2) to determine their possible role in the renoprotective effect exerted by BB in this study. We found a downregulation of the genes (ACE and AT1) in the vasoconstrictor arm of the RAS, and a

concomitant upregulated expression of genes belonging to the vasodilatory arm of RAS, namely ACE2 and AT2, in the kidney of BB-fed OZR rats, when compared to the control OZR rats. These findings suggest that BB-exerted renoprotective effect can be attributed partly to improved RAS components in the kidney and indicates yet another pleiotropic effect of BB.

Oxidative stress induced generation of reactive oxygen species (ROS) and other free radicals has been implicated in MetS [33, 34], pathogenesis of kidney diseases [35-37] and also in MetS-induced tubulointerstitial injury [38]. Superoxide anions have been shown to cause increased smooth muscle cell contraction by regulating cytosolic calcium concentrations [39]. Our EPR data show that the generation of ROS, superoxide and peroxynitrite were increased in the medulla and cortical tissue of the kidneys of MetS animals. This increase in the mediators of oxidative stress in the kidney tissue can induce vasoconstriction of the arterioles, thus contributing to the decreased RBF and elevated RVR in the MetS animals [40]. Further, the antioxidant defense mechanism in these OZRCC animals were weak compared to the LZRCC and LZRBB rats, as indicated by the SOD and catalase enzyme activity. The BB-enriched diet, however, was able to restore the redox balance in the MetS animals.

Obesity and hypertension are hallmarks of MetS that have been implicated in kidney diseases. In addition to the injury to the kidney tissue [41], proteinuria and microalbuminuria have been linked to MetS-associated CKD [27, 42, 43]. Studies have also shown that glomerulopathy [41] and injury-induced reduction in the absorption by the tubules result in proteinuria [44]. Our histopathological data clearly indicates a marked disruption of the renal structure in the OZRCC animals compared to the LZR rats. This structural damage in OZRCC animals included glomerular sclerosis, interstitial nephritis, fibrosis and proteinuria. We also examined the gene expression of TGF- β , which is an important profibrotic marker [45]. The

OZRCC animals had an elevated expression of TGF- β (compared to LZRCC and LZRBB) which was significantly reduced in the OZRBB group. Interestingly, previous studies have correlated renal TLR4 expression with the inflammatory marker TGF- β in CKD [46]. An increase in TGF- β mRNA levels has been implicated in the glomeruli of diabetic rats [47]. Further, we also measured the albuminuria levels in these animals and our results were consistent with the histopathological findings. BB was able to exert a reno-protective effect by attenuating nephropathy and albuminuria in OZRBB rats.

Inflammation is one of the major contributors to the progression of MetS-induced kidney disorders [48, 49]. TLR4 signaling pathways and their expression have been studied in relation to inflammation in kidney injury [6, 50]. We examined the gene and protein expression of TLR4 in the kidney cortical tissues of animals from all experimental groups. Interestingly, the gene and protein expression of TLR4 was significantly increased in the OZRCC rats compared to the LZRCC and LZRBB groups. The TLR4 signaling-driven inflammation is, at least in part, a possible cause for the progression of glomerular and tubular injury in the MetS animals, thereby contributing to renal dysfunction. The MetS animals that were fed on a BB-diet had a reduced gene and protein expression of TLR4 in the kidney cortex, thus indicating a protective role of BB in the kidney of these animals.

Further, to elucidate the downstream mechanism of reno-protection by BB, we examined the association of TLR4-mediated MAPK activation in MetS-induced kidney dysfunction. Previous findings have established a potential link between ERK1/2 phosphorylation and oxidative stress-induced insulin resistance [19], which is a characteristic of MetS. Xi L. et al recently showed that selective inhibition of ERK1/2 in rat hepatocytes improved impaired insulin signaling [51]. In addition, inhibition of p38MAPK signaling pathways has been shown to

prevent diet-induced MetS in rats [52]. Although MAPK activation has been shown in LPS-induced kidney dysfunction [17, 18, 53], a role for the TLR4-MAPK pathway in MetS-induced CKD has never been investigated. Our findings demonstrate phosphorylated-ERK and p38MAPK in OZRCC animals compared to the LZRCC and LZRBB. Interestingly, BB-treated OZR -had suppressed ERK and p38MAPK activity in the kidneys. This inhibition of MAPK activation in OZRBB animals could also be a plausible explanation for the improved glucose sensitivity in these animals. Therefore, these results not only designate a key role of TLR4-MAPK signaling in MetS-associated CKD but also indicate a potential mechanism by which BB protects against chronic kidney injury.

In addition to attenuated TLR4 and MAPK expression, OZRBB exhibited decreased expression of PICs. We examined the expression of gene and protein expression of IL-1 β and IL-18 in the kidney cortex of animals from all experimental groups. The importance of IL-1 β and IL-18 expression patterns in this study is particularly important, not only because these are proinflammatory molecules, but also as these are downstream molecules of the TLR4 pathway. We observed that OZRBB animals expressed a significantly lower amount of IL-1 β and IL-18 compared to the OZRCC rats. This is consistent with the previous findings from our lab, which reported that in SHR, a renal function decline involves an association of PICs with their transcription factor NF κ B [3]. Further, oxidative stress is a key regulator of NF κ B activity [54] and it produces a positive feedback mechanism related to inflammation and tissue injury. In this context, we also measured the NF κ B activity in all the groups, as NF κ B activity is an indicator of TLR4 activation. Our data shows that BB attenuates the NF κ B activity in MetS rats, thus confirming the reno-protective effect of BB is, at least in part, mediated by TLR4.

NFκB and MAPK pathways regulate the expression of many genes involved in inflammation and tissue injury [55, 56] and their inhibition has been shown to have tissue protective effects. The Nrf2 antioxidant pathway is a key mechanism in the attenuation of kidney injury [57] by reno-protective agents [58, 59]. Several mechanisms of Nrf2 activation have been reported, including MAPK signaling pathways. In normal physiological conditions, the MAPK family maintains the much needed balance between NFκB and Nrf2 activation [60]. In pathological conditions though, the pro-oxidative NFκB arm dominates and contributes to the increased generation of pro-inflammatory cytokines and ROS, thus weakening the anti-oxidant defense system. Nrf2 and Keap1 protein expression were examined in the cytoplasmic extracts. Nrf2 regulates the expression of several anti-oxidant enzymes within the cell. Although Keap1 is known to regulate the cytoplasmic-nuclear shuttling of Nrf2, it also controls the degradation of Nrf2 [61]. A higher expression level of Nrf2, concomitant with a reduced expression level of Keap1 in the cytoplasmic fraction indicates increased cyto-protective activity. Therefore, we assessed the expression levels of Nrf2 and Keap1 in the cytoplasmic fraction. Our data indicate that BB intervention inhibits TLR4 in chronic kidney disease, thereby augmenting the anti-inflammatory actions of Nrf2 over the pro-inflammatory actions of NFκB. In light of these findings, we underline the anti-inflammatory and antioxidant properties of BB, wherein the reno-protective effect could be due to a suppression of inflammation and subsequent increase in anti-oxidant mechanism or vice versa.

In summary, our results suggest a major role for blueberry in protecting against MetS-associated CKD via a decrease in inflammation and most importantly, this study provides comparative evidence for the mechanism of action of BB, via inhibition of TLR4, and consequent attenuation of ERK and p38MAPK phosphorylation. This study also suggests that

non-pharmacological antioxidant approaches have protective value against MetS-associated CKD and also open up the possibility of a potential target in TLR4 against renal diseases.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Ms. Sherry Ring for her technical assistance.

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CHAPTER 5

SUMMARY AND CONCLUSIONS

5.1 OVERALL SUMMARY OF FINDINGS

The incidence of renal diseases is on the rise in the United States and statistics show that more than 20 million American adults have some kind of kidney diseases (1). If left untreated, these acute and chronic kidney disease conditions might progress to End Stage Renal Disease (ESRD). End Stage Renal Disease still remains a leading cause of mortality and morbidity in the United States (2). An important molecule that is upstream of several inflammatory cascades, TLR4, has been associated with the injurious effects of inflammation on the renal structure and function (3-5). Although previous studies have proposed a vital role for TLR4 in acute and chronic renal diseases, the mechanisms by which TLR4 exerts its inflammatory effect on the kidneys are still poorly understood. The overall aim of this dissertation was to elucidate the signaling mechanisms by which TLR4 contributes to the development of acute and chronic kidney diseases. We further hypothesized that pharmacological and non-pharmacological approaches that inhibit the expression of TLR4 would prevent or delay the progression of renal diseases.

In Chapter 2, we determined the molecular mechanism by which AngiotensinII (AngII) induces renal injury by modulating TLR4 signaling using an *in vitro* model of rat tubulo-epithelial cell line. AngII treatment in NRK52E cells induced an increase in the expression of inflammatory molecules such as TLR4 and HMGB1, along with increased expression of proinflammatory cytokines TNF α and IL-1 β . We showed that pretreatment with losartan (an AT1 receptor blocker) attenuated the AngII-induced expression of TLR4 and inflammatory cytokines. TLR4 gene silencing was used to confirm that AngII-induced inflammation was

mediated through TLR4 in these cells. TLR4siRNA treatment in these cells significantly decreased the expression of proinflammatory cytokines and HMGB1. Further we treated the cells with anti-HMGB1 to investigate if the AngII mediated effect was modulated by HMGB1-TLR4 signaling. Cells pre-treated with anti-HMGB1 before AngII exposure also expressed an attenuated inflammatory profile as seen with TLR4-silenced cells. Downstream activation of NF κ B and rate of generation of ROS were also decreased on gene silencing of TLR4 and/or exposure to anti-HMGB1. These findings indicate a key role for TLR4 signaling in AngII-mediated inflammation in the renal epithelial cells. Our data also reveal that AngII-induced effects could be alleviated by HMGB1-TLR4 inhibition, suggesting this pathway as a potential therapeutic target for hypertensive renal dysfunctions.

In Chapter 3, we focused on an *in vivo* acute kidney injury (AKI) model to elucidate the effect of TLR4 in AKI. We used lipopolysaccharide (LPS) to induce AKI. More importantly, LPS is a specific ligand for TLR4. We also used blueberry (a rich source of antioxidants and possessing anti-inflammatory properties) supplementation as a potential non-pharmacological approach to attenuate the expression of TLR4 and study if blueberry (BB) supplementation could prevent or reduce the incidence of LPS-induced AKI. Male Sprague-Dawley rats received a BB solution or saline intra-gastric gavage for 2 days. One group of BB and saline gavaged animals was injected with LPS (10mg/kg b.w). Another group of rats was injected with VIPER (0.1mg/kg i.v), a TLR4 specific inhibitory peptide, 2 hours before LPS administration. Compared to LPS-administered rats, the BB pretreated animals exhibited improved glomerular filtration rate, elevated renal blood flow, and a reduced renal vascular resistance. In addition, a reduction in the rate of production of free radicals, namely total reactive oxygen species (ROS) and superoxide, was observed in the BB supplemented LPS group. Gene and protein expression

for TLR4, proinflammatory cytokine and acute kidney injury markers were also attenuated in animals that were pretreated with BB as measured by real time RT-PCR and western blotting respectively. The results in the BB pretreated group were consistent with the VIPER treated rats. These results highlight a role for TLR4 in LPS-induced AKI, and suggest that BB, by inhibiting TLR4 and its subsequent effect on inflammatory and oxidative stress pathways, is a viable non-pharmacological option to decrease AKI.

In Chapter 4, we examined the effect of TLR4 signaling in an animal model of metabolic syndrome-associated chronic kidney disease (CKD) and investigated if a blueberry-enriched diet could attenuate the progression of CKD in this animal model. We extended our study to further investigate the downstream mechanism of TLR4 signaling that contributes to the progression of CKD in these animals. We tested the hypothesis that inflammation-induced renal damage is triggered by the activation of TLR4, and subsequent modulation of redox-sensitive molecules and the mitogen-activated protein kinase (MAPK) pathway. Five-week old lean and obese zucker rats (LZR and OZR) were fed a BB-enriched diet or an isocaloric control diet for 15 weeks. We showed that control OZR exhibited lower glucose tolerance, exacerbated renal dysfunction, and increased oxidative stress. Gene and protein expression levels of TLR4 were higher and this was accompanied by increased renal pathology with extensive albuminuria and deterioration in antioxidant levels in OZR. In addition, OZR had increased phosphorylation of ERK and p38MAPK. BB-fed OZR exhibited significant improvements in all these parameters compared to OZR. These results suggest that the TLR4-MAPK signaling pathway is a key to the renal structural injury and dysfunction in MetS and BB protect against this damage by inhibiting TLR4. This is the first study to demonstrate a potential mechanism of TLR4-induced kidney

damage in a model of MetS and elucidate a downstream mechanism by which BB exert their reno-protective effects.

5.2 SIGNIFICANCE OF THE RESEARCH AND FUTURE DIRECTIONS

Despite the recent advances in its treatment, renal diseases still remain a leading cause of mortality and morbidity in the United States (2). Oxidative stress (6, 7), inflammation and inflammation-associated diseases such as hypertension (8-10), diabetes (11, 12) are major contributors to the development of kidney disorders and its progression to end stage renal disease. Currently available antioxidative and anti-inflammatory therapies modulate oxidative stress and inflammation in the kidney but their efficacy in treating renal diseases is marginal at best. The increased rate of incidence of renal diseases in the United States warrants the need for new strategies for the treatment of acute and chronic renal diseases.

Recent evidence indicates an important role for TLR4 in inflammation and inflammatory diseases. However, the effect of TLR4 activation on acute and chronic kidney diseases is still poorly understood. In addition, the signaling mechanism for the TLR4-associated inflammatory effect in renal diseases has not been elucidated. Thus, a better understanding of the causative role of TLR4 in the development of renal diseases is important for the identification of novel therapeutic targets.

Our studies have focused on elucidating the mechanisms by which TLR4 modulates the inflammatory cascade and thereby contributes to the progression of renal diseases in different models of acute and chronic kidney disease. We have shown that targeting TLR4, both pharmacologically and non-pharmacologically, is an effective method for attenuating renal injury and the progression of kidney disease.

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APPENDIX
LETTER OF PERMISSION

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Apr 06, 2015

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Expected completion date	May 2015
Estimated size (number of pages)	100
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
VAT/Local Sales Tax	0.00 USD / 0.00 GBP
Total	0.00 USD

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