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Evaluating Cecal Ligation And Puncture- Induced Kidney Injury In Diabetic Meprin Deficient Mice

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Biographical Sketch

Kasheena Burris is a native of Los Angeles, CA. She was born on April 23rd, 1989 in Compton, CA, the middle child of her parents Kathia Brown and Gerald Burris. Kasheena is a loving sibling to her sisters Kyisha and Melissa Burris, and her brothers Ricky Roberts, and Ryan and Armond Burris. At the age of thirteen Kasheena and her sister Kyisha moved in with their aunt Sandra Williams in Los Angeles, CA. During her middle school years Kasheena played basketball and the violin. Kasheena always had a love for sports. While in high school Kasheena participated in Med Core Scholars at the University of Southern California as well as the Summer Enrichment Program at Pomona College. It was through those programs that she became interested in the biomedical sciences.

She began her college career studying laboratory animal sciences in 2007 at North Carolina Agricultural and Technical State University in Greensboro, NC. During her undergraduate career she worked in the Laboratory Animal Resource Unit and as an undergraduate research assistant. She learned to properly handle and treat research animals and conducted research alongside animal science graduate students. In 2011 she graduated with her Bachelor's degree in Laboratory Animal Science. In the fall of 2012, Kasheena enrolled in the MS Biology program at North Carolina Agricultural & Technical State University. She worked as a laboratory instructor for an undergraduate-level course (BIOL 100), where she provided support for the course lecturer. Kasheena's thesis research was supported by a Basic Immune Mechanisms Training Grant from the National Institutes of Health.

Dedication

This thesis is dedicated to my family and close friends, those who always believed in me.

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Abbreviations and Symbols

α Greek Letter Alpha

β Greek Letter Beta

µg Micrograms

µl Microliter

µm Micrometer

°C Degrees Celsius

ARF Acute Renal Failure

ANOVA Analysis of Variance

BBM Brush-Border Membrane

BUN Blood Urea Nitrogen

CO_2 Carbon Dioxide

CLP Cecal Ligation and Puncture

ddH_2O Distilled Deionized Water

DN Diabetic Nephropathy

ECM Extracellular Matrix

ELISA Enzyme-Linked Immunosorbent Assay

EDTA Ethylenediamine Tetra-acetic Acid

ESRD End Stage Renal Disease

FDA Food and Drug Administration

g Gram

G Gauge

GFR Glomerular Filtration Rate

IgG Immunoglobulin G

HEPES 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

HRP Horseradish Peroxidase

kg Kilogram

KO Knockout

LPS Lipopolysaccharide

mg Milligram

ml Milliliter

M Molar

mM Millimolar

mRNA Messenger Ribonucleic Acid

MyD88 Myeloid Differentiation Factor 88

NaCl Sodium Chloride

Na_3VO_4 Sodium Orthovanadate

PAGE Polyacrylamide Gels Gel Electrophoresis

PBS Phosphate-Buffered Saline

RIPA Radioimmunoprecipitation Assay

RNA Ribonucleic Acid

SDS Sodium Dodecyl Sulfate

SNPs Single Nucleotide Polymorphisms

STZ Streptozotocin

x g Relative Centrifugal Force

TBS Tris-Buffered Saline

TBS-T Tris-Buffered Saline with Tween 20

TEMED Tetramethylethylenediamine

TGF-β1 Transforming Growth Factor Beta 1

Abstract

Diabetic nephropathy (DN) is the leading cause of end stage renal disease (ESRD), and is associated with high morbidity and mortality rates. Key histological changes observed in DN include accumulation of extracellular matrix (ECM) proteins and tubulointerstitial fibrosis. Meprins are metalloproteinases that are abundantly expressed in the brush border membranes of proximal kidney tubules. Meprins are also expressed in leukocytes (monocytes and macrophages) and podocytes. Meprins cleave/degrade extracellular matrix (ECM) proteins such as collagen IV, collagen VI, fibronectin, laminin, and nidogen-1 *in vitro.* Meprins have been implicated in the pathology of DN. Sepsis is a complex medical condition, where the entire body undergoes an inflammatory state and the presence of a known or suspected infection leads to severe consequences such as multiple organ failure. Acute renal failure (ARF) is a common complication of sepsis. The objective of this study was to evaluate cecal ligation and puncture (CLP)-induced sepsis in meprin deficient mice with type 1 diabetes as a co-morbidity. Low dose Streptozotocin (STZ) was used to induce type-1 diabetes in wild-type (WT) C57BL/6 mice which express high levels of both meprin A and meprin B, and meprin α knockout mice on a C57BL/6 background, which are deficient in meprin A. Cecal ligation and puncture was performed 4 weeks post STZ injection. Blood was collected pre and post CLP to evaluate blood urea nitrogen (BUN) levels. The mice were sacrificed 18hr post CLP and kidney tissue processed for proteomic analysis. BUN levels were significantly higher in CLP mice and meprin α knockout mice had lower mortality rates in comparison to wild-type mice. The results show that meprin deficiency protected mice from kidney injury associated with CLP, suggesting that meprins play a role in kidney injury following CLP-induced sepsis.

CHAPTER 1

Introduction

Diabetes is the most rampant endocrine disease and affects millions of Americans alone. Diabetic nephropathy (DN) is one of the major microvascular complications of diabetes and it is associated with a rise in the urinary albumin excretion (UAE) rate and abnormal renal function. Currently, changes in albuminuria are considered a hallmark of onset or progression of diabetic nephropathy. Approximately 20–30% of patients with type 1 or type 2 diabetes develop evidence of nephropathy (Ailing Lu and Anupam Agarwal 2011). Diabetic nephropathy is the leading cause of end stage renal disease (ESRD). Meprins are metalloproteinases that are highly expressed in the brush border membranes (BBM) of proximal kidney tubules (Bond & Beynon, 1995; Kounnas et al., 1991). The self-associating homo-oligomeric complexes of meprin A are secreted as latent proteases (containing the prosequence) and can move through extracellular spaces in a non-destructive manner, and deliver a concentrated form of this metalloproteinase to sites that have activating proteases, such as site of inflammation, infection or cancerous growth. Thus, meprin structures provide means to concentrate proteolytic activity at the cell membrane (Bond & Beynon, 1995; Kounnas et al., 1991). Meprins have been shown to cleave ECM proteins such as collagen IV, collagen VI, fibronectin, laminin, and nidogen-1 *in vitro* (Banerjee & Bond, 2008; Kaushal et al., 1994; Kohler et al., 2000; Kruse et al., 2004).

Sepsis is a complex medical condition, where the entire body undergoes an inflammatory state and the presence of a known or suspected infection leads to severe consequences such as multiple organ failure (Bone et al., 1992). Acute renal failure (ARF) is a common complication of sepsis and carries an ominous prognosis. Mortality was reported higher in patients with septic ARF (74.5%) than in those whose renal failure did not result from sepsis (45.2%) (Vriese,

2003a). Although studies have been conducted, the exact pathogenesis of diabetic nephropathy is complex and not completely understood. Identifying anomalies of kidney function in the early stages of diabetic nephropathy is vital to developing an ideal treatment and cure. The objective of this research was to evaluate the role of meprins in the kidney injury associated with CLP-induced sepsis using a meprin α deficient mouse model.

CHAPTER 2

Literature Review

2.1 Diabetes

Diabetes is a chronic systemic disease characterized by high levels of glucose in the blood. Types 1 and 2 diabetes mellitus together affect more than 20 million Americans and rank as the sixth leading cause of disease-related death in the United States (Abdin et al., 2010; A. Red Eagle et al., 2005; Wada & Makino, 2013). People with diabetes have high blood glucose levels due to either the lack of insulin production by the pancreas or the inability of cells to process insulin. Patients with high blood sugar will typically experience polyuria (frequent urination), they will become increasingly thirsty (polydipsia) and hungry (polyphagia). In the body, the metabolic hormone insulin is imperative to blood glucose homeostasis. Insulin is produced by pancreatic β cells in the body(H.M. Wagner E., Bloom D., and Camerini D, 1998).

The onset of type-1 diabetes is directly linked to the malfunction of these pancreatic β cells in the body. People usually develop type 1 diabetes before their 40th year, often in early adulthood or teenage years. Type 1 diabetes requires treatment with insulin or transplantation of pancreatic b cells. Patients with type 1 diabetes will need to take insulin injections for the rest of their life(H.M. Wagner E., Bloom D., and Camerini D, 1998). They must also ensure proper blood-glucose levels by carrying out regular blood tests and following a special diet. By contrast, the majority of the diabetic population has type 2 diabetes, which is not insulin-dependent. In most cases this form of diabetes does not require insulin therapy. Type 2 diabetes is characterized by persistent hyperglycemia, impaired glucose tolerance, glomerular hyperfiltration, and progression of albuminuria, ultimately leading to renal injury(Schena, 2005).

The prevalence of diabetes is projected to increase from 171 million in 2000 to 366 million in 2030, as a result of growth, aging, urbanization, and physical inactivity (Wild et al., 2004). Type-1 and type-2 diabetes continue to increase on an epidemic scale and have become a major public health concern globally. Identifying new therapeutic targets are critical to suppressing this epidemic.

2.1.2 Complications of Diabetes Diabetes is associated with several complications. These include: weight loss, polyuria, hypertension, blurred vision and diabetic nephropathy (DN).

2.2 Diabetic Nephropathy

Diabetic nephropathy is one of the most severe microvascular complications of diabetes mellitus and is also a major cause of end-stage renal disease(Schena, 2005). Diabetic nephropathy is associated with albuminuria, proteinuria and reduction in glomerular filtration rate (Ching Ye Hong, 1998). The elevated levels of serum creatinine and blood urea nitrogen are considered to be an index of diabetic nephropathy. In the glomeruli, mesangial cells are considered to be a primary target for the insult induced by increased glomerular capillary pressure and play a crucial role in the glomerular trafficking of plasma proteins, their deposition, and extracellular matrix (ECM) protein accumulation within the mesangium (Luca Paris, 2008). This leads to the development and progression of glomerular sclerotic lesions in various glomerular diseases such as diabetic nephropathy. The thickening of basement membranes in capillaries and small vessels of diabetic patients is considered to be a characteristic histological finding in diabetic nephropathy (Rafel Sim, 1996).

Ethnic disposition to ESRD varies greatly across different racial groups. In figure 1 shown below, both the rates of incident of ESRD caused by diabetes and their growth over time

vary widely by age and race/ethnicity (Health, 2013). Among whites age 30–39, for example, the rate (adjusted for gender) has increased just 3.5 percent since 2000, reaching 37 per million in 2011. For blacks/African Americans of the same age, in contrast, the rate has increased 72 percent since 2000, to reach 136 per million. Different patterns are seen among older populations in the same figure. The 2011 rate of incident ESRD due to diabetes among whites age 50–59 is nearly the same as in 2000, while rates have fallen 27 and 50 percent, respectively, among blacks/African Americans and Native Americans of the same age(Health, 2013). The wide variation of incidence of ESRD caused by diabetes is not fully understood.

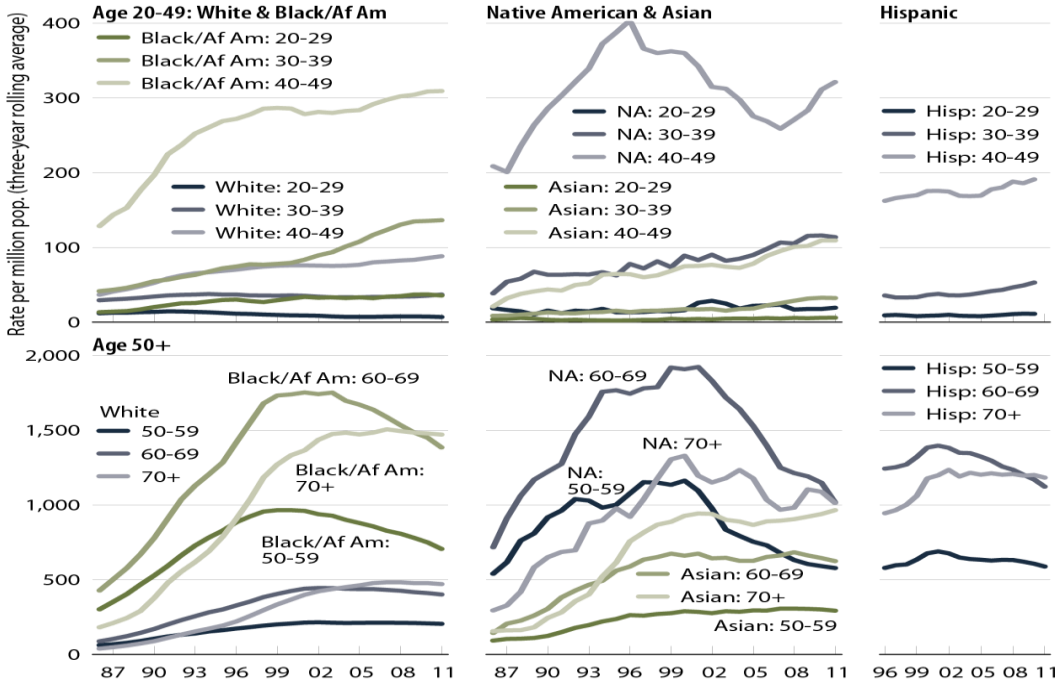

Figure 1. Adjusted incident rates of ESRD due to diabetes, by age, race, & ethnicity.

2.2.1 Histology of Diabetic Nephropathy Diabetic nephropathy development is characterized by the progressive change in kidney function. This change occurs in series of stages. During the initial stages, diabetics experience hyperglycemia and glomerular hyperfiltration(Ayo, 1990). Subsequent stages include thickening of the glomerular basement membrane, mesangial cell expansion, proteinuria, acute and severe hypertension, and the

eventual decline in glomerular filtration rate (GFR). These stages ultimately lead to end stage renal disease (ESRD) (Ching Ye Hong, 1998). It should also be noted that the loss of renal functionality leads to a decline in nephrons and accumulation of extracellular matrix (ECM). ECM abundance has been linked to upregulation of transforming growth factor-β (TGF-β), a fibrogenic cytokine (Eddy & Neilson, 2006; Lan, 2011). Production of TGF-β stimulates ECM synthesis while inhibiting degradation (Lan, 2011). Treatment with anti-TGF-β in db/db mice, a mouse model of type-2 diabetes showed decreased glomerular basement membrane thickening and mesangial matrix accumulation (Chen et al., 2003). Extracellular matrix buildup in the diabetic kidney surpasses degradation and initiates glomerulosclerosis and tubulointerstitial fibrosis (Chen et al., 2003).

Hyperglycemia also causes an increase in the synthesis of fibronectin, laminin, and type IV collagen in glomerular mesangial cells. Laminin, an adhesive glycoprotein, is the main non-collagenous constituent of the basement membrane and is up regulated in diabetic patients (Rafel Sim, 1996). In recent years, biochemical and immunohistochemical approaches have been developed to characterize the changes of laminin in basement membrane and alterations of the metabolism and distribution of this protein have been described in diabetic animals and also in humans (Rafel Sim, 1996). Fibronectin is involved in coagulation, platelet formation, tissue repair, and may reduce erythrocyte deformity and filterability in diabetic patients (Ching Ye Hong, 1998). Glomerular mesangial cells are considered to be exposed to the stretch stress due to glomerular hypertension and are found to produce the excess amount of extracellular matrix (ECM) proteins including fibronectin when exposed to the mechanical stretch. This is important because increases in mesangial cell

proliferation and extracellular matrix proteins, such as fibronectin , ultimately result in thickening of the glomerular basement membrane (Ailing Lu & Anupam Agarwal, 2011). Understanding the pathogenesis of diabetic nephropathy, along with its complications, in the early stages is necessary to develop targeted therapies to detect alterations in kidney function. It remains to be fully defined as to which pathways in diabetic complications are essentially protective rather than pathological, in terms of their effects on the underlying disease process. Today, clinical indicators of diabetic nephropathy include blood urea nitrogen (BUN), proteinuria, serum creatinine, and glomerular filtration rate (GFR) measurements to asses kidney function(Bluestone, 2010).

2.2.2 Role of Mesangial Cells in Diabetic Nephropathy In the glomeruli, mesangial cells are considered to be a primary target for the insult induced by increased glomerular capillary pressure and play a crucial role in the glomerular trafficking of plasma proteins, their deposition, and extracellular matrix (ECM) protein accumulation within the mesangium (Luca Paris, 2008). Secretion of ECM proteins by mesangial cells could thus, lead to the development and progression of glomerular sclerotic lesions in various glomerular diseases such as DN. The thickening of basement membranes in capillaries and small vessels of diabetic patients is considered to be a characteristic histological finding in DN (Rafel Sim, 1996).

Fibronectin is involved in coagulation, platelet formation, tissue repair, and may reduce erythrocyte deformity and filterability in diabetic patients (Ching Ye Hong, 1998). It is believed that mesangial cell proliferation and extracellular matrix accumulation play crucial roles in early renal hypertrophy and later glomerular sclerosis in diabetic nephropathy. Mesangial cells excrete more extracellular proteins under high glucose conditions, but the mechanism behind this is not understood. Glomerular mesangial cells are considered to be exposed to the stretch stress due to

glomerular hypertension and are found to produce the excess amount of extracellular matrix (ECM) proteins including fibronectin and laminin when exposed to the mechanical stretch (Luca Paris, 2008). This is important because increases in mesangial cell proliferation and extracellular matrix proteins, including fibronectin , ultimately result in thickening of the glomerular basement membrane (Ailing Lu & Anupam Agarwal, 2011). This ultimately leads to impaired renal function. Previous studies have demonstrated that high glucose levels stimulated mesangial cell proliferation and fibronectin expression leading to extracellular matrix deposition.

Laminin, an adhesive glycoprotein, is the main non-collagenous constituent of the basement membrane and is up regulated in diabetic patients. In recent years, biochemical and immunohistochemical approaches have been developed to characterize the changes of laminin in basement membrane and alterations of the metabolism and distribution of this protein have been described in diabetic animals and also in humans (Rafel Sim, 1996). The most widely used marker for laminin metabolism in humans is the LPl fragment and its assay in serum has proved useful in the monitoring of patients with malignancies and liver disease. These pathways ultimately lead to increased renal albumin permeability and extracellular matrix accumulation, resulting in increasing proteinuria, glomerulosclerosis and ultimately tubulointerstitial fibrosis (Luca Paris, 2008).

2.2.3 Signaling Pathways Involved in Extracellular Matrix Metabolism The protein kinases regulate a series of cellular processes during growth and development. Protein kinases are an integral part of the machinery that is activated in response to stress, they are essential for memory, and they are directly involved in orchestrating cell death (S.S. Taylor, 2006). These enzymes are primary targets for therapeutic intervention. Examining the localization and signaling between these protein kinases will provide some explanation in the hyper-excretion of

these extracellular basement proteins which ultimately lead to renal fibrosis and diabetic nephropathy. Several treatment strategies are available to cure diabetic nephropathy or reduce its progression. These include modalities used to suppress the renin–angiotensin–aldosterone system and control blood glucose levels (Eades, 2009). However, diabetic patients are still reaching end stage renal disease at an alarming rate. Conventional therapeutic strategies are not fully efficacious in the treatment of diabetic nephropathy, suggesting an incomplete understanding of the gene regulation mechanisms involved in its pathogenesis (Eades, 2009).

2.2.4 Sepsis and the Immune Response Sepsis is a complex medical condition, where the entire body undergoes an inflammatory state and the presence of a known or suspected infection leads to severe consequences such as multiple organ failure (Bone et al., 1992). Sepsis serves as diabetes most common co-morbidity. Acute renal failure (ARF) is a common complication of sepsis and carries an ominous prognosis. Mortality was reported higher in patients with septic ARF (74.5%) than in those whose renal failure did not result from sepsis (45.2%) (Vriese, 2003b). Inflammatory cells infiltrate the kidney, causing local damage by release of oxygen radicals, proteases, and further production of inflammatory cytokines. Cytokines act as polypeptides regulating inflammatory and immune responses through actions on cells (Hewlett M. Wagner E., Bloom D., and Camerini D, 2004). Inflammatory cytokines, mainly IL-1, IL-6, and IL-18, as well as TNF-α, are involved in the development and progression of diabetic nephropathy (Vriese, 2003b). As shown below in figure 2, the three significant steps in sepsis are vasodilation and vascular leak, leukocyte recruitment, and coagulation and neutrophil extracellular trap (NET) formation. Local inflammatory mediators, including tumor necrosis factor (TNF) and interleukin (IL)-1β, lead to vasodilation (Hewlett M. Wagner E., Bloom D., and Camerini D, 2004). This recruits leukocytes to sites of infection and sets off a

cascade of leukocyte activation, NET formation, and coagulation (Vriese, 2003b). The recognition of these molecules as significant pathogenic mediators in diabetic nephropathy leaves open the possibility of new potential therapeutic targets (Hewlett M. Wagner E., Bloom D., and Camerini D, 2004). Those suffering from systemic sepsis suffer from acute lung injury, acute kidney injury, and even death.

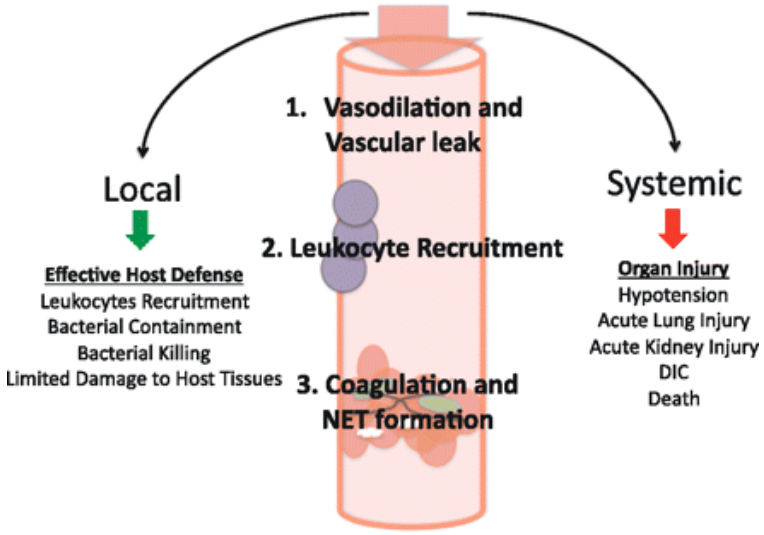

Figure 2. Schematic of Sepsis.

People with diabetes may also be at increased risk of developing acute renal failure (ARF). The presence of underlying diabetic nephropathy may predispose to ARF resulting from adverse effects such as hypotension, sepsis or exposure to nephrotoxic agents (Ching Ye Hong, 1998). Understanding the role that the immune system plays in the pathogenesis of diabetic nephropathy could lead to identification of new strategies and/or additional therapeutic targets for prevention and treatment of diabetic nephropathy (Hewlett M. Wagner E., Bloom D., and Camerini D, 2004).

2.3 Meprins

Meprins are cell surface and secreted proteases highly expressed in the brush border membranes of proximal kidney tubules (Bertenshaw et al., 2001; Bond, Matters, Banerjee, & Dusheck, 2005; Kounnas, Wolz, Gorbea, & Bond, 1991). Meprins are members of the astacin 8 family of metalloproteases (Bond & Beynon, 1995). Meprin A and meprin B are disulfide-linked, tetrameric metalloendopeptidases in renal brush border membranes. Meprins are highly expressed at the brush border membrane of proximal tubule cells of the kidney and epithelial cells of the intestine (Carlos M. Gorbea, 1991). Meprin proteases are composed of two evolutionarily related subunits, α and β, that are approximately 50% identical at the amino acid level. The subunits are encoded on two genes: the α gene is on human chromosome 6 (mouse 17) near the histocompatibility complex; the β subunit on chromosome 18 in both the mouse and human genomes (Judith S. Bond, 2005). The self-associating homo-oligomeric complexes of meprin A are secreted as latent proteases (containing the prosequence) and can move through extracellular spaces in a non-destructive manner, and deliver a concentrated form of this metalloproteinase to sites that have activating proteases, such as site of inflammation, infection or cancerous growth (Carlos M. Gorbea, 1991). In situ hybridization studies of embryonic and adult mice and immunohistochemistry demonstrated the tissue-specific expression of meprin subunits in the epithelial cells of kidney and intestine only. Kidney expressions of mouse meprin subunits are strain-dependent; all strains express both subunits during fetal stages. Some strains increase both subunits after birth (e.g., C57BL/6) while others only express meprin β, and down regulate mepin α (e.g., C3H/He) (Judith S. Bond, 2005). Meprin structures provide means to concentrate activity at the cell membrane.

Researchers also determined the tissue-specificity of meprin subunits in embryonic and adult mice. The study established that the expression of kidney meprin subunits is dependent on the strain of mice; C57BL/6 mice expresses both α and β meprin subunits whereas adult C3H/He lack the meprin α subunit (Kumar & Bond, 2001). Within the kidney, meprin expression is normally restricted to the brush-border membrane, however when injury occurs, meprins are transferred to other cell compartments. This transfer of meprins to other cellular compartments can increase damage to kidney tissue.

2.3.1 Meprins Structures Meprins are composed of two subunits, alpha (α) and beta (β), that are evolutionarily related but differ in function and structure (Bond & Beynon, 1995; Wolz & Bond, 1995). The α and β subunits are encoded by two distinct genes on chromosomes 6 and 18 in humans and 17 and 18 in mice (Bond, Rojas, Overhauser, Zoghbi, & Jiang, 1995). Meprin A consists of homo-oligomeric α/α complexes and hetero-oligomeric α/β complexes, while meprin B is a homo-oligomer of β/β complexes (Beynon, Oliver, & Robertson, 1996; Bond & Beynon, 1995; Gorbea et al., 1993). Meprin β subunits are integral membrane proteins that consist of a short cytoplasmic tail and a trans-membrane domain (Johnson & Hersh, 1994; Marchand, Tang, & Bond, 1994). The meprin subunits form homo or hetero complexes linked by disulfide bonds and can be expressed separately or coordinately (Bond et al., 2005). When the subunit α is associated with a β subunit, it remains attached to the cell membrane.

2.3.2 Meprins Substrates Meprins are highly conserved among different species and are capable of degrading a wide range of proteins, such as ECM proteins collagen IV, collagen VI, fibronectin, laminin, and nidogen-1 *in vitro* (Banerjee & Bond, 2008; Kaushal, Walker, & Shah, 1994; Kohler, Kruse, Stocker, & Sterchi, 2000; Kruse et al., 2004). Both α and β subunits have specific substrates that are capable of being degraded such as bradykinin (Bertenshaw, Villa,

Hengst, & Bond, 2002) for meprin A and gastrin (Bertenshaw et al., 2001) , orcokinin (Bertenshaw et al., 2002), pro-inflammatory cytokines (Marchand et al., 1994; Norman, Matters, Crisman, & Bond, 2003), E-cadherin (Huguenin et al., 2008) and protein kinases (Chestukhin, Litovchick, Muradov, Batkin, & Shaltiel, 1997) for meprin B. Meprins also cleave parathyroid hormone (Yamaguchi, Fukase, Sugimoto, Kido, & Chihara, 1994), biologically active peptides (Kohler et al., 2000; Sterchi, Naim, Lentze, Hauri, & Fransen, 1988) and chemokines. The localization of meprins at the interface with the external environment, at leukocytes at inflammatory sites, and in response to bacterial infections implicates them in host defense.

2.3.3 Meprins and Diabetic Nephropathy Meprins have been linked with a variety of pathological conditions such as ischemia-reperfusion, induced acute renal failure, diabetic nephropathy, and fibrosis. Other studies demonstrate that low levels of meprin A are associated with the development of chronic nephropathy and fibrosis in animal models of diabetes (Bond et al., 2005; Mathew et al., 2005). Researchers used polymerase chain reaction to determine variations in the meprin β gene in Pima Indians, a Native American tribe with significantly high rates of type 2 diabetes and diabetic nephropathy (A. R. Red Eagle et al., 2005). This critical study revealed 19 single nucleotide polymorphisms (SNPs) in the meprin β gene, suggesting that there are genes that make individuals susceptible to diabetic nephropathy (A. R. Red Eagle et al., 2005). Urine samples were collected from premenopausal women with histories of urinary tract infections (Bond et al., 2005). Researchers found that women with acute urinary tract infections had high or very high levels of meprin in the urine (Bond et al., 2005).

2.3.4 Meprins and Cecal Ligation and Puncture Induced Sepsis Sepsis is a disorder initiated by excessive activation of innate immunity. It is a serious medical problem particularly in patients in the intensive care unit (ICU) where it is the second leading cause of death in non-

coronary ICU patients. Acute renal injury (AKI) occurs in 20%–50% of septic patients and nearly doubles the mortality rate of sepsis (A. R. Red Eagle et al., 2005). There is a growing recognition of the need for treatment regimens that target both the early systemic and later kidney-specific effects of sepsis in patients. Meprins are also capable of proteolytically processing cytokines and chemokines (Hewlett M. Wagner E., Bloom D., and Camerini D, 2004). For example, meprin A and meprin α are capable of generating biologically active IL-1β from its precursor pro-IL-1β (Herzog C, 2009). Recent studies have demonstrated that meprin-α knockout mice were protected against lipopolysaccharide (LPS)-induced AKI. This finding supported a recent study shown that actinonin administered at the time of induction of sepsis by cecal ligation and puncture (CLP) in mice reduced renal injury (Holly MK, 2006). However, this study did not address the mechanism of protection. Since current therapy is mostly supportive and largely ineffective there is a critical need to uncover new therapeutic approaches because the incidence of sepsis-induced AKI is predicted to increase as the population ages (Jandeleit-Dahm, 2006).

CHAPTER 3

Methodology

3.1 Reagents

The following chemicals were purchased from Sigma-Aldrich (St Louis, MO): mannitol, sodium citrate, sodium chloride, sodium dodecyl sulfate, streptozotocin (STZ) tetramethylethylenediamine (TEMED) and triton X-100. The following chemicals were purchased from Fisher Scientific (Pittsburgh, PA): β-mercaptoethanol, acetic acid, ammonium persulfate, choloroform, ethylenediaminetetraacetic acid (EDTA), EZ-Run pre-stained rec protein ladder, fat-free milk, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), isobutyl alcohol, hydrochloric acid, magnesium chloride, methanol, sodium orthovanadate, tris base, and tween 20. The following chemicals were purchased from Bio-Rad (Hercules, CA): anti-mouse IgG secondary antibody, anti-rabbit secondary antibody, Bio-Rad's protein reagent, 30% acrylamide (29:1 bis solution). Dr Judith Bond from Pennsylvania State University College of Medicine (Hershey, PA) donated anti-meprin-A polyclonal rabbit and anti-meprin-B polyclonal rabbit antibodies. Anti-PKA mouse monoclonal antibody was purchased from BD Biosciences (Greensboro, NC). The following chemicals were purchased from Thermo Scientific (Waltham, MA): ethyl alcohol 200 proof (Acros Organics), 100X EDTA solution, 100X halt protease inhibitor cocktail and West Pico ® chemiluminescent substrate.

3.2 Experimental Animals

Wild-type male mice on a C57BL/6 background were purchased from Charles River Laboratories (Wilmington, MA). Meprin α knockout (αKO) mice on a C57BL/6 background were bred in Laboratory Animal resource Unit (LARU) at North Carolina Agricultural & Technical State University. All mice were housed in standard cages with 5 mice per cage with a

12:12 hour light:dark cycles and were fed a standard mouse chow (Purina Laboratory Chow 5001; Purina Mills, St Louis, MO) and water ad libitum. All animal protocols were approved by the North Carolina A&T State University Institutional Animal Care and Use Committee (IACUC).

3.3 Induction of Diabetes in Mice

Low dose streptozotocin (STZ) was used to induce type-1 diabetes in 8 week old mice. STZ was dissolved in sodium citrate buffer (10 mmol/L, pH 4.5) to make a stock of 7.5 mg/mL. STZ was used within 15 minutes of preparing and kept from light to avoid degradation. Mice were injected with STZ at 55 mg/kg using a 29G insulin needle, to induce type-1 diabetes. Control mice were injected with equivalent volumes of sodium citrate buffer. Injections were repeated for 5 consecutive days. All mice were fasted for 6 hours prior to injections. Mice were weighed prior to injections and every week thereafter. Blood glucose levels were measured for each mouse at 10 days post-STZ injections using a Reli-On® Blood Glucose Monitoring System (ARKRAY USA, Minneapolis, MN). STZ-injected mice with a blood glucose level >250 mg/dL were considered diabetic.

3.4 Cecal Ligation and Puncture

Mice were anesthetized with Ketamine (100 mg/kg) and Xylazine (100 mg/kg) dissolved in 0.9 % sterile saline solution intraperitoneal. The lower abdominal quadrant of each mouse was shaved using an electric trimmer and disinfected with Betadine and Nolvasane 3x. Applied eye ointment to the eyes and monitored the intensity of anesthesia by a toe pinch. A midline longitudinal incision was made to exteriorize the cecum and contents within the cecum were pushed toward the blind end ~15cm from end. The ligated cecum was perforated by a single through-and-through puncture with a 21-gauge needle and squeezed to extrude a 1 mm column

of fecal material. In sham-operated mice, the cecum was located, but neither ligated nor punctured. The cecum was relocated into the abdominal cavity. The abdominal incision was closed in two layers with 5-0 nylon sutures running sutures. After surgery, 1 ml of pre-warmed 0.9% sterile saline solution was injected intraperitoneally. Mice were allowed to recover then returned to cages with food and water on pre-warmed deltaphase isothermal pads. Six hours post-CLP mice were given Buprenorphrine (0.3 mg/ml) dissolved in 0.9 % sterile saline solution intraperitoneal for pain.

3.5 Tissue Collection and Analysis

Blood and urine samples were collected at 4 weeks post-STZ injections. Blood samples were collected from each C57BL/6 mouse by nicking the tail vein and drawing into lithium/heparin tubes (Sarstedt, Newton, NC), which prevent clotting of the blood. Blood was collected at 4 weeks post-STZ injections for both male and female mice. Blood samples were centrifuged at 10000 x g for 10 minutes at 4˚ C. Plasma was stored at -80 ˚C until used for kidney assessments. Spot urine samples were collected by bladder massage. To harvest kidney tissue, the mice were put to death by inhalation of CO_2. Both left and right kidneys were removed and decapsulated. Half of the kidney was wrapped in aluminum foil, snap-frozen in liquid nitrogen and stored at -80 ˚C for proteomic analysis. Other sections of each kidney were cut and placed into Carnoy's fixative (60% Methanol, 30% Chloroform, 10% Acetic Acid) overnight. The kidney sections were then removed from the fixative and stored in 70% Ethanol at 4˚C. Kidney tissue was embedded in paraffin embedded and 5 μm cross sections were cut onto Superfrost plus microscope slides (Fisher Scientific, Pittsburgh, PA) for histology.

3.6 Blood Urea Nitrogen Assay

To evaluate kidney function, blood urea nitrogen (BUN) was measured at 4 weeks post-STZ injections using the stored plasma samples. BUN was assessed using BUN chemistry slides (Ortho Clinical Diagnostics, Rochester, NY) and then analyzed on the Vitros DT6011 Analyzer (Ortho Clinical Diagnostics, Rochester, NY).

3.7 Fractionation of Kidney Proteins

Mice kidneys, previously stored at -80 ˚C were thawed and fractionated into cytosolic-, brush border membrane- and structural-enriched fractions. Additionally, samples containing total protein content were obtained. Kidneys were homogenized in 9 volumes of Kidney Brush Border Homogenization Buffer (2mM Tris HCl, pH 7.0 with 10 mM Mannitol). A 1 M stock of MgCl2 was added for a final concentration of 10 mM and stirred at 4 ˚C for 14 minutes. The homogenate was centrifuged at 15000 x g at 4 ˚C for 15 minutes and the supernatant was transferred to a new microcentrifuge tube. The supernatant was centrifuged at 15000 x g at 4˚C for 15 minutes; afterward the supernatant was transferred into a new microcentrifuge tube and stored at -80 ˚C as the cytosolic-enriched fraction. The pellet was resuspended in 500 µL Kidney Brush Border Homogenization Buffer and centrifuged at 2200 x g at 4 ˚C for 15 minutes. The supernatant was centrifuged at 2200 x g for 15 minutes and discarded. The pellet was resuspended in 100 µL Kidney Brush Border Homogenization Buffer and stored at -80 ˚C as the brush border membrane-enriched fraction. Radioimmunoprecipitation Assay buffer (RIPA) (0.02 mM HEPES pH 7.9, 0.015 mM NaCl, 0.1 mM Triton-X 100, 0.01 mM SDS, 1 mM Na3VO4) with 10% 0.5 M EDTA was used to extract total protein mix and kept on ice for 30 minutes. The homogenate was centrifuged at 16100 x g at 4 ˚C for 10 minutes and supernatant was transferred into a new microcentrifuge tube then stored at -80 ˚C. Supernatant is the total-enriched fraction.

Proteins concentrations from all fractions were determined by the Bradford Reagent Protein Assay Method, using Bio-Rad's Protein Assay Reagent (Hercules, CA).

3.8 Western Blot Analysis

Western blot analysis was used to quantify the kidney protein levels of meprin A, meprin B and the catalytic subunit of protein kinase A (PKAcat). Equal amounts of kidney protein (30-80 μg) were loaded into 10% prepared Sodium Dodecyl Sulfate Polyacrylamide Gels (SDS-PAGE) and allowed to separate by electrophoresis for 1 hour at 200 Volts. Proteins from the gels were transferred to nitrocellulose membrane (Bio-Rad, Richmond, CA) using a Trans-Blot SD Semi-Dry Transfer Cell Unit (Bio-Rad, Richmond, CA). To block nonspecific binding sites, membranes were incubated in 8% fatty-free milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 1 hour at room temperature with gentle shaking. Nitrocellulose membranes were incubated with primary antibodies overnight at 4˚C or at room temperature for 1 hour. The primary antibodies used were polyclonal rabbit anti-meprin α and β (Hershey Medical Center, Hershey, PA), diluted 1:5000 and mouse monoclonal anti-PKAcat (BD Biosciences Greensboro, NC), diluted 1:3300. The nitrocellulose membranes were washed three times for 10 minutes at room temperature. The secondary antibody mouse IgG (Bio-Rad, Hercules, CA) or rabbit IgG (Bio-Rad, Hercules, CA) was added to the nitrocellulose membranes using a dilution of 1:10,000 overnight at 4˚C or at room temperature for 1 hour. The nitrocellulose membranes were washed three times for 15 minutes at room temperature. Nitrocellulose membranes were then exposed to Chemiluminescent Substrates (Thermo Scientific, Waltham, MA) and developed on X-Ray film. Protein bands were evaluated by densitometry using QuantityOne Software (Bio-Rad, Hercules, CA).

3.9 Statistical Analysis

The data were analyzed by two-way ANOVA, with Bonferroni post-test pair-wise comparisons using Graph Pad Prism Software (GraphPad Software, La Jolla, CA). P values ≤ 0.5 were considered statistically significant.

CHAPTER 4

Results

4.1 Blood Glucose

Diabetes was confirmed by measuring blood glucose ten days post-STZ injections for each mouse. Mice with a blood glucose reading > 250 mg/dL are considered diabetic. Blood glucose levels for STZ-injected WT and meprin αKO mice were significantly higher in comparison to sodium citrate (NaC) buffer injected mice. Wild-type and meprin αKO mice subjected to CLP surgery had higher glucose levels than sham operated controls.

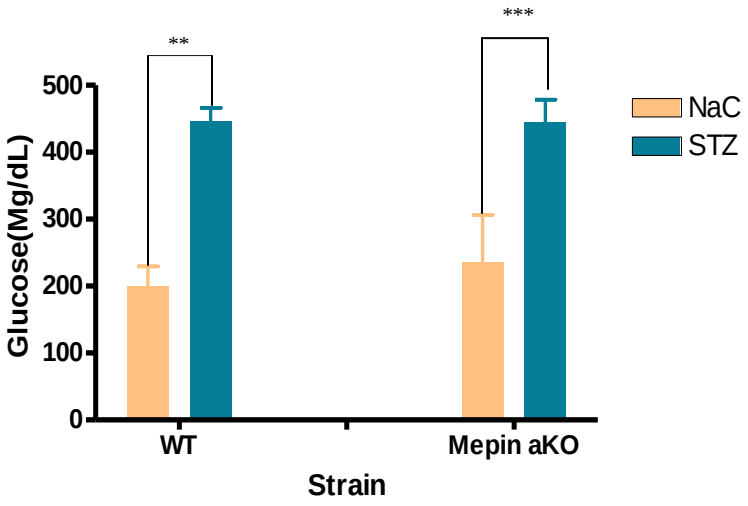

Figure 3. WT and αKO 10 Day Glucose Measurements (**= P<0.01;***=P<0.001)

4.2 WT Pre- vs. Post-Diabetic Body Weights

Body weights were collected at 0 and 4 weeks post STZ injection in WT mice examine the effects of diabetes on body weight.

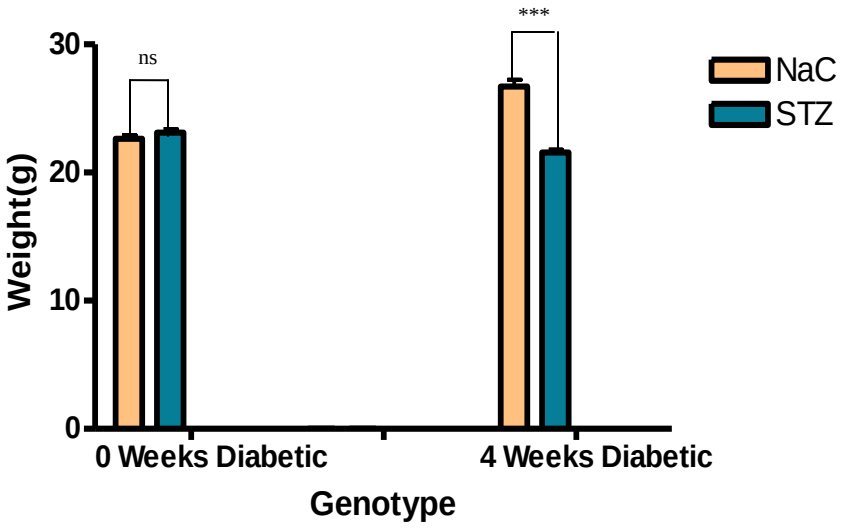

Figure 4. WT body weights 0 weeks diabetic vs. 4 Weeks diabetic. (ns= P<0.05;***=P<0.001) .

4.3 WT BUN Levels Pre- vs. Post-CLP

Plasma samples were processed 0 and 18 hours post CLP to assay BUN in C57BL/6 (WT) mice. BUN levels were higher in the WT mice 18 hours post CLP surgery.

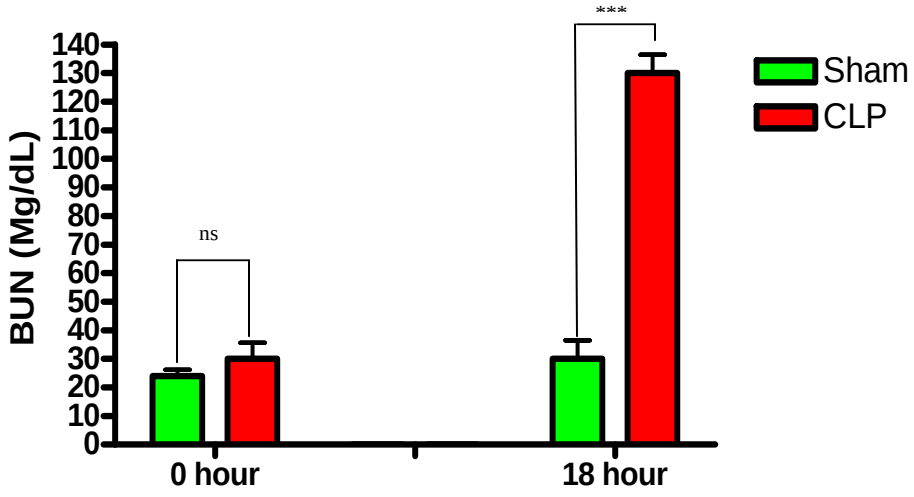

Figure 5. WT BUN levels 0 hour and 18 hour CLP (ns= P<0.05;***=P<0.001)

4.4 αKO BUN Pre- vs. Post-CLP

Plasma samples were processed 0 and 18 hours post CLP to assay BUN in αKO mice. BUN levels were higher in the αKO mice 18 hours post-CLP surgery.

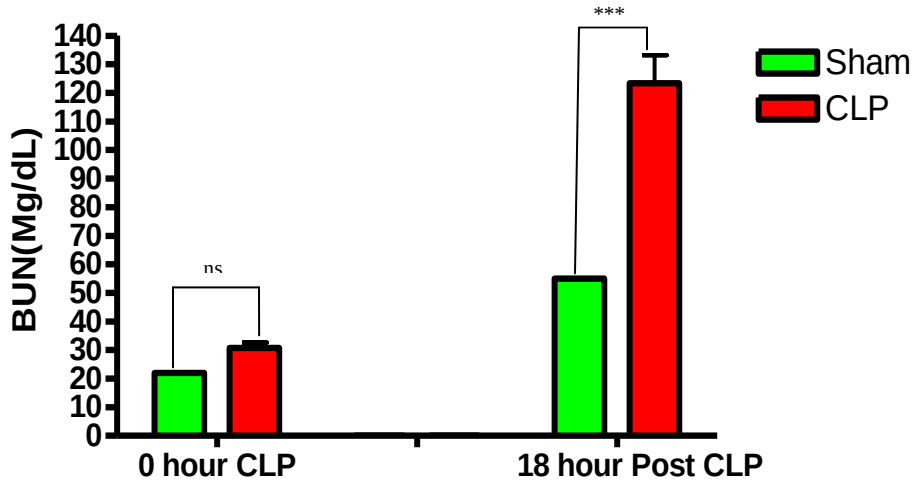

Figure 6. αKO BUN levels 0 hour and 18 hour CLP (ns=P<0.05;**=P<0.01)

4.5 WT BUN levels Mon-diabetic vs. Diabetic

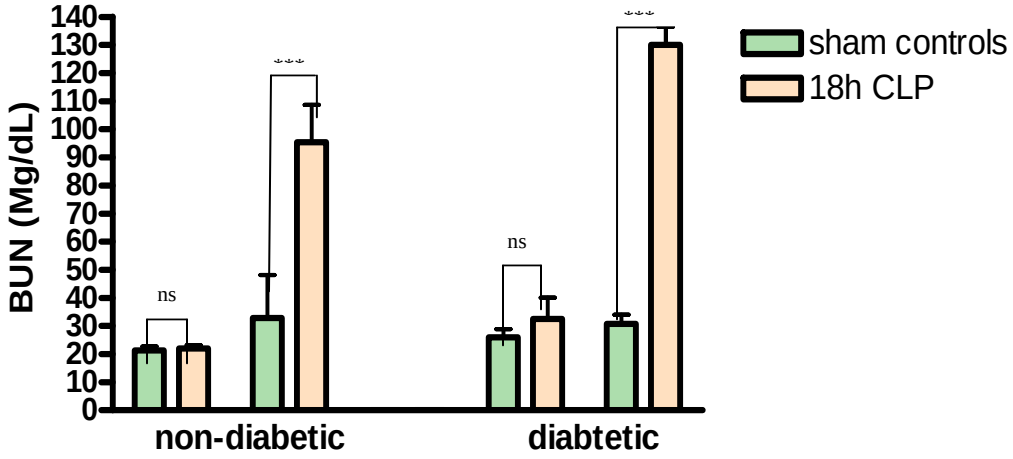

Figure 7. WT BUN levels non-diabetic vs. diabetic (ns= P<0.05;***=P<0.001).

4.6 Serum Creatinine Levels

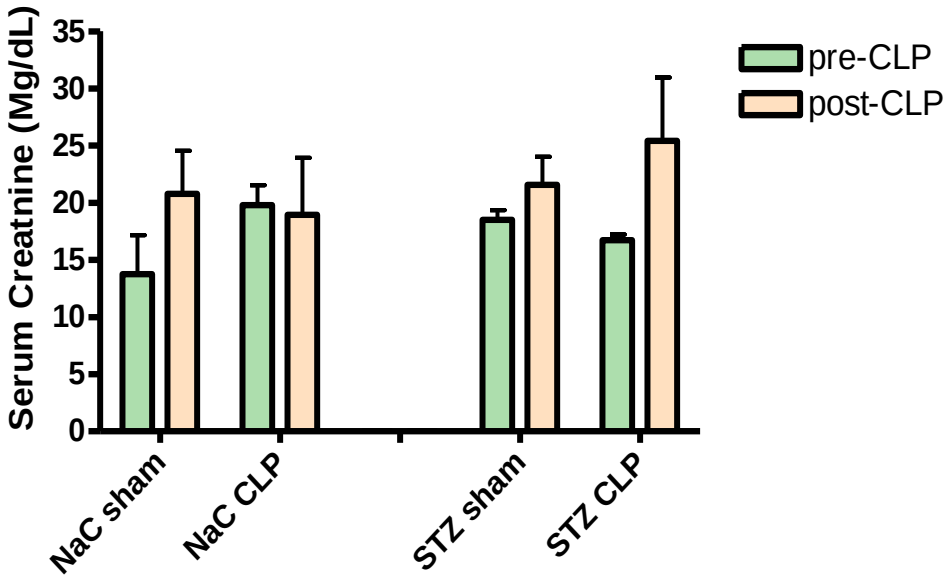

Figure 8. Serum creatinine levels in WT mice pre and post CLP.

4.7 CLP Mortality Rates

Mortality rates were evaluated for both the sham and CLP induced sepsis mice. 30% of mice that had undergone CLP induced sepsis died overnight in comparison to 10% of the sham mice.

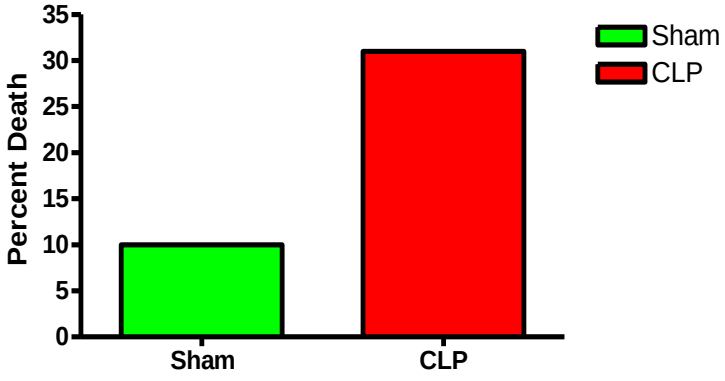

Figure 9. CLP was associated with a high mortality rate.

4.8 Genotype CLP Mortality Rates

Mortality rates were determined by genotype. 20% of the C57BL/6 (WT) mice died 18 hours post CLP surgery in comparison to 10% of αKO mice.

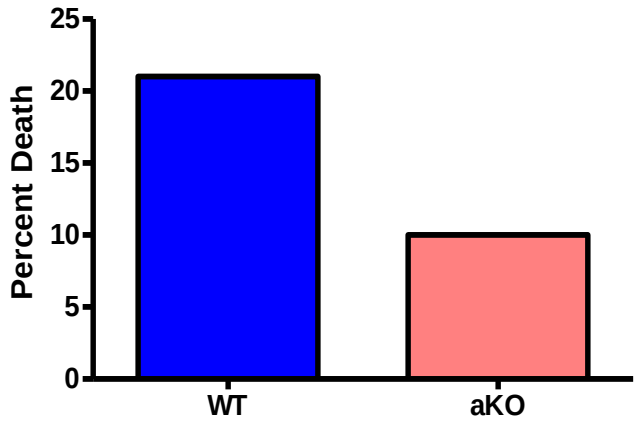

Figure 10. Meprin A deficiency decreased CLP-associated mortality

CHAPTER 5

Discussion and Future Research

Diabetic nephropathy is the leading cause of ESRD worldwide (Ibrahim & Hostetter, 1997, Molitch et al., 2003; A. R. Red Eagle et al., 2005; Thrailkill et al., 2009). It has become an increasing concern in medicine because it is associated with high mortality and morbidity rates. Pathological changes observed in diabetic nephropathy include: accumulation of ECM proteins, proteinuria, renal hypertrophy, glomerular basement membrane thickening, mesangial expansion and renal fibrosis (Lan, 2011; Maxwell, 2005; Wada & Makino, 2013). Meprin metalloproteases are abundantly expressed in the BBM of the kidney and have been shown to degrade ECM proteins. Sepsis is a disorder initiated by excessive activation of innate immunity. It is a serious medical problem particularly in patients in the intensive care unit (ICU) where it is the second leading cause of death in non-coronary ICU patients. There is a growing recognition of the need for treatment regimens that target both the early systemic and later kidney-specific effects of sepsis in patients. Meprins are also capable of proteolytically processing cytokines and chemokine. For example, meprin A and meprin α are capable of generating biologically active IL-1β from its precursor pro-IL-1β (Herzog C, 2009). Recent studies have demonstrated that meprin-α knockout mice were protected against lipopolysaccharide (LPS)-induced AKI (Hewlett M. Wagner E., Bloom D., and Camerini D, 2004).

Data from this study suggests that meprin deficient mice that had undergone CLP induced sepsis had less severe kidney damage than WT mice in comparison to their sham counterparts. Both WT and αKO mice had higher BUN levels post CLP surgeries. STZ-injected WT mice had higher BUN levels post CLP in comparison to the control group.

The mechanisms by which meprins protect against sepsis in diabetic nephropathy are not fully understood. A potential pathway is through modulation of ECM metabolism. Further studies need to be done with meprin knockout mice to further evaluate mechanisms by which meprins protect mice from diabetic kidney damage in septic conditions. Having a suitable model for DN is critical in advancing research in DN and ultimately identifying biomarkers that can be used for early diagnosis of patients at risk for DN. Early diagnosis is important in providing targeted treatments to patients and decreasing both mortality and morbidity rates.

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