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# Evaluating The Interaction Between Protein Kinase C And Meprins In Diabetic Nephropathy

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# Evaluating the interaction between Protein Kinase C and Meprins in Diabetic Nephropathy

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## Biographical Sketch

Sada Boyd was born in Detroit, MI on November 5th, 1990 to James and Romilda Boyd. She has two older brothers, Antonio Terry and Paris Terry. As a child she played trumpet and sang in the choir. While in high school, Sada was a member of the National Honor Society and participated in the Gaining Early Awareness and Readiness for Undergraduate Programs (Gear-UP). Participation in the Gear-UP program afforded her the opportunity to go on multiple college tours. On one of those tours, Sada found her undergraduate institution, Bennett College for Women.

While attending Bennett College, Sada majored in Biology and received various scholarships and awards. Bennett College is where she met many of her close friends and mentors. During her sophomore year at Bennett College, she became a member of Louis Stokes Alliance for Minority Participation (LSAMP). Participation in LSAMP encouraged her to participate in two summer research programs for undergraduates. These research experiences inspired her to pursue a career as a research scientist. From 2011-2012, she served as the president of Bennett College Choir.

In the Fall 2012, Sada enrolled as a Biology Master's student at North Carolina Agricultural and Technical State University in Greensboro, NC. While at North Carolina A&T, she was under the supervision of Robert Newman and Elimelda Ongeri. During her time spent in the Master's program, she learned various laboratory skills. Sada is grateful for the education she received during her matriculation at NC A&T.

# Dedication

This thesis is dedicated to all of the people who encouraged me throughout my life.

# Acknowledgments

I would like to acknowledge my research advisor, Dr. Robert Newman, for his patience and guidance during my time at North Carolina Agricultural and Technical State University. He has truly been a great mentor and advisor. He has supported and encouraged me throughout my time here at NC A&T. Present and former members of the Newman lab: Tiffany Kennedy, Micheal Furse, David Clarke, Carl Parson and Shante Hutchinson, who assisted with various experiments. I would like to thank my co-advisor, Dr. Ongeri, for encouraging me to pursue my career aspirations and pushing me to think critically. She has been a great mentor and advisor who has inspired me. Former members of the Ongeri lab: Barry Martin, Sabena Conley, Kasheena Burris, Jasmine George who assisted in teaching me various laboratory techniques. Present members of Ongeri lab, Wy-Key Brower, Regine Harris and Corshe Stanley, who have assisted with various experiments. I want to thank Jean-Marie Niyitegeka, who has been very helpful in the completion of my thesis. I am grateful to Dr. Patrick Martin, who served on my committee and helped during my matriculation through the Master's program. Additionally, I would like to thank the Biology Department and the BEACON foundation for financial support. Lastly, I would like to thank James and Romilda Boyd, Paris Terry, Antonio Terry and Doupa Kpangbai they have been very supportive throughout my educational experience.

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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

BBM Brush-Border Membrane

$CO_2$ Carbon Dioxide

dd$H_2O$ Distilled Deionized Water

DN Diabetic Nephropathy

ECM Extracellular Matrix

ESRD End Stage Renal Disease

FDA Food and Drug Administration

g Gram

IgG Immunoglobulin G

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

HRP Horseradish Peroxidase

kg Kilogram

KO Genetic knockout

LPS Lipopolysaccharide

mg Milligram

ml Milliliter

## Abstract

Meprin metalloproteinases have been shown to play a role in the progression of diabetic nephropathy (DN) in both mice and humans. However, the mechanisms involved are not fully understood. Protein Kinase C (PKC) family members are a group of phospholipid-dependent serine/threonine kinases that regulate signaling pathways involved in cellular growth, proliferation, differentiation, and cell death. PKC signaling pathways have also been implicated in diseases such as DN. Specifically, PKCα has been shown to play a role in the development of albuminuria in mice with streptozotocin (STZ)-induced type 1 diabetes. The objective of this study was to determine whether PKCα is a meprin substrate. To this end, activated forms of purified recombinant meprin A and B were incubated for 0-4 h with cytosolic-enriched proteins from either protein lysates extracted from Mardin-Darby canine kidney (MDCK) cells (which do not express meprins) or meprin αβ double knockout mouse kidneys (which lack endogenous meprins). The levels of PKCα were determined by Western blot analysis using anti-PKCα specific antibodies. Incubation with meprin B significantly reduced the levels of PKCα present in the kidney and MDCK lysates. This decrease was not observed in proteins incubated with meprin A or control reactions lacking meprins, suggesting isoform-specific degradation of PKCα. To confirm the degradation, human PKCα was expressed in yeast, purified by GST-affinity chromatography, and incubated with the activated meprins. To determine whether meprin cleavage of PKCα occurs in models of diabetes, immunohistological analysis was conducted using mouse kidney tissues from STZ-diabetes induced mice. These data suggest that meprin B may impact kidney function via proteolysis of PKCα.

# CHAPTER 1

## Introduction

Diabetes mellitus is a chronic disorder caused by an inherited and/or acquired deficiency in the production of insulin by the pancreas and/or by resistance to insulin's effects (Evicmen & King, 2007). Diabetes and its complications affect many people worldwide, with a greater prevalence in developed countries. The number of people in North America who suffer from diabetes has increased substantially over the past two decades. Moreover, the increased blood glucose levels, or hyperglycemia, associated with diabetes causes damage to many body systems, including the eyes, nerves, and kidneys. Indeed, chronic hyperglycemia is believed to be the major cause of several diabetic complications, such as hypertension, diabetic nephropathy (DN), diabetic retinopathy (DR), and impaired wound healing. Previous studies in the United Kingdom have demonstrated that treatment that lowers blood glucose reduces the risks of diabetic retinopathy, nephropathy, and neuropathy (American Diabetes Association, 2002).

Diabetic nephropathy (DN), which is kidney disease associated with long-standing diabetes, is the leading cause of end-stage renal disease (ERSD). It is the most common cause of progressive chronic kidney disease and ESRD in the Western world (Menne, 2013). Activation of Protein Kinase C (PKC) by hyperglycemia has been implicated in the induction of DN (Geraldes & King, 2010). Indeed, PKC isoform-selective inhibitors represent promising new therapeutics that have been shown to delay the onset or even stop the progression of diabetic vascular disease with very few side effects (Evicmen & King 2007).

PKC, a founding member of the PKA/PKG/PKC (AGC) family of serine/threonine protein kinases, plays a key role in a variety of cellular functions by regulating many signal transduction pathways (Newton, 2003). PKC is activated by elevated levels of second

messengers, such as diacylglycerol (DAG) and calcium ions. The PKC family can be divided into three major sub-groups based on their mode of activation. These include the conventional PKCs (cPKC), which are regulated by both $Ca^{2+}$ and DAG, the novel PKCs (nPKC), which are regulated by DAG but are insensitive to $Ca^{2+}$, and the atypical PKCs (aPKC), which are insensitive to both DAG and $Ca^{2+}$. Each group contains specific isoforms that are believed to have distinct cellular functions. This study focuses on the conventional PKC isoform, PKCα. PKCα can be activated by many stimuli, including mechanical strain, physical stress, such as hypoxia, and receptor activation (Nakashima, 2002). PKCα has been implicated in the pathogenesis of DN (Geraldes, 2010). The inappropriate activation of PKCα during DN is the impetus for this study.

Meprins are metalloproteinases that are highly expressed at the brush border membrane (BBM) of kidney epithelial cells (Norman, 2003). Meprins are also expressed in podocytes, skin, and leukocytes (Ongeri, 2012). They are known to degrade extracellular matrix proteins (Dimas, 2013) and have been implicated in the progression of kidney injury. In addition to their role at the membrane, meprins are able to redistribute from the BBM to the cytosol and basolateral membranes of kidney proximal tubular cells under conditions of ischemia reperfusion (Bylander, 2008; Ongeri, 2011). Importantly, the redistribution of meprins to the cytosol appears to have functional consequences.

Previous studies in our lab demonstrated that the catalytic subunit of another AGC family member, the alpha-isoform of the catalytic subunit of Protein Kinase A (PKA Cα), is cleaved by meprin metalloproteinases in an isoform-specific manner (Niyitegeka, in press, AJP-Renal). For instance, meprin B cleaves PKA Cα at both its N- and C-terminus while meprin A fails to cleave PKA Cα. The C-terminal cleavage site of meprin B, which reduces the catalytic activity of PKA

Cα nearly 20-fold, is conserved among several AGC family members, including PKCα (Figure *1).*

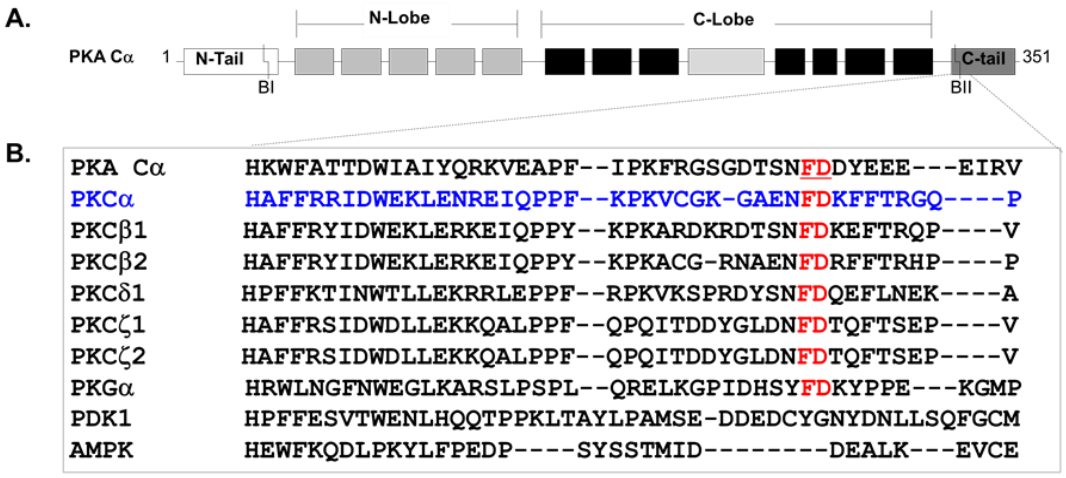


*Figure 1.* Conservation of Meprin B Cleavage Site among AGC family Members. (A) Schematic diagram showing the domain structure of the catalytic subunit of PKA (PKA Cα) and sites of Meprin B cleavage at the N- and C-termini (BI and BII, respectively). (B) Multiple sequence alignment of several AGC family members. Site BII in (A) (highlighted in red), is conserved in many PKC isoforms, including PKCα, the focus of this study (highlighted in blue).

# CHAPTER 2

# Literature Review

## 2.1 Diabetes

Diabetes mellitus is a chronic disease caused by an inherited and/or acquired deficiency in the production of insulin by the pancreas (type 1 diabetes) or by resistance to insulin's effects (type 2 and gestational diabetes) (Evcimen & King, 2007). Diabetes, which is characterized by hyperglycemia due to insulin deficiency, affects nearly 170 million people worldwide and is expected to double by 2030 (Geraldes & King, 2010). More than 80 % of diabetes cases are found in developed countries. There are three types of diabetes: type 1, type 2, and gestational diabetes. Type 1 diabetes is an autoimmune disease that results in the destruction of insulin-producing pancreatic beta cells, leading to insulin deficiency. Type 2 diabetes, which affects 80-90 % of all diabetes patients, is caused by insulin resistance. Finally, gestational diabetes is caused by insulin resistance that is first seen during pregnancy (Venkat-Narayan, 2006).

Many complications stemming from diabetes have been observed. Such diabetes-associated disorders are the result of interactions among a number of systemic metabolic abnormalities, including hyperglycemia, dyslipidemia, disruption of genetic/epigenetic modulators, and local tissue responses to toxic metabolites (Geraldes & King, 2010). For instance, DN is the major cause of end-stage renal disease (ESRD) in the developed world, accounting for 40 % to 50 % of all documented cases (Molitch, 2008).

Chronic hyperglycemia is considered one of the major causes of diabetic complications. Macrovascular (involving large blood vessels) and microvascular (involving small blood vessels) complications are particularly prevalent in those patients with type 2 diabetes. Several organs, such as muscle, skin, brain, heart and kidneys, are affected by macrovascular and microvascular

complications attributed to diabetes (Cade, 2008). These complications involve large vessel obstructions, such as coronary artery diseases, atherosclerosis, and peripheral vascular diseases, as well as microvascular pathologies, including retinopathy, neuropathy and nephropathy (Geraldes & King, 2010).

## 2.2 Diabetic Nephropathy

Diabetic nephropathy (DN) is a progressive and serious complication of diabetes caused by angiopathy that damages the kidneys, which can ultimately lead to renal failure. DN is characterized by a set of structural and functional hallmarks that occur in diabetic patients, including proteinuria, cellular hypertrophy and increased extracellular matrix protein production (Reeves & Andreoli, 2000). DN is the leading cause of ESRD, with a high mortality and morality rate in the United States, accounting for almost 40 % of all new dialysis patients (Reeves & Andreoli, 2000). Certain populations, such as American Indians, Hispanics and African Americans, are at greater risk of developing DN than Caucasians (Reeves & Andreoli, 2000). Interestingly, patients with type 2 diabetes have a higher occurrence rate of DN compared to those with type 1 diabetes.

### 2.2.1 Complications due to diabetic nephropathy

A set of structural and functional abnormalities in the kidneys of diabetic patients characterize DN. DN is first characterized by microalbuminuria, which worsens to overt albuminuria followed finally by acute renal failure (ARF) (Cade, 2008). In diabetic kidney disease, cellular hypertrophy, thickening of the glomerular basement membrane (GBM) and tubular basement membrane (TBM) occur (Wada & Makino, 2013). During DN, production of ECM surpasses degradation, causing ECM proteins to build up in the glomerular and tubulointerstitial compartments and to manifest as glomerulosclerosis and tubulointerstitial

fibrosis in the affected kidney (Wada & Makino, 2013). Recent evidence suggests that ECM accumulation is linked to elevated levels of transforming growth factor-β (TGF-β) in the diabetic kidney, which is caused by high glucose levels (Lee, 2013).

The cellular and structural changes outlined above lead to a number of functional alterations in DN. Functional alterations include an early increase in the glomerular filtration rate with intraglomerular hypertension, subsequent proteinuria, systemic hypertension, and eventual loss of renal function (Reeves & Andreoli, 2000). One in four people with type 2 diabetes have microalbuminuria or a more advanced stage, which worsens at a rate of 2% a year (Cade, 2008). Although there has been extensive research on this complication of diabetes, its pathophysiology is not fully understood.

### 2.2.2 Signaling pathways associated with diabetic nephropathy

Diabetic nephropathy (DN) is characterized by a plethora of abnormalities, which lead to progressive albuminuria, podocyte loss, progressive glomerular sclerosis and tubulointerstitial fibrosis associated with the disease (Brosius, 2010). For instance, glucose-dependent pathways that are activated within the diabetic kidney include those that lead to increased oxidative stress, renal polyol formation and glycated end-products (Cooper, 2001). There are three major pathways associated with the development of DN: (i) the activation of polyol and PKC pathways; (ii) the formation of advanced glycation end-products; and (iii) intraglomerular hypertension induced by glomerular hyperfiltration (Wada & Makino, 2013).

Haemodynamic factors are also known to have implications in the pathogenesis of DN. These include increased systemic and intraglomerular pressure and activation of various vasoactive hormone pathways, including the renin-angiotensin system and endothelin (Cooper, 2001). Signaling enzymes, such as PKC and MAP kinase, as well as their downstream effectors,

such as TGF-beta, can become activated by haemodynamic pathways either independently or in the context of metabolic pathways. These pathways lead to increased renal albumin permeability and extracellular matrix accumulation, which results in increasing proteinuria, glomerulosclerosis and tubulointerstitial fibrosis (Cooper, 2001), all of which are characteristics of DN. Since there are multiple factors that contribute to the pathogenesis of DN, it is difficult to establish a hierarchy of signaling pathways involved in the disorder (Brosius, 2010).

### 2.2.3 Intersection between the PKC signaling pathway and diabetic nephropathy

There are a host of diabetic legions associated with DN. For instance, hyperglycemia, glycosylated proteins, intracellular polyols, vasoactive hormones, systemic and glomerular hypertension, proteinuria, growth factors, and cytokines have all been implicated in the pathogenesis of DN (Reeves & Andreoli, 2000). These mediators may lead to the inappropriate activation of PKC. Inappropriate activation of PKC and its isoforms alter insulin action on blood vessels, causing vascular abnormalities and pathologies. Hyperglycemia may be the most important factor in causing these vascular abnormalities and is considered to be the driving force of ESRD (Wada & Makino, 2013).

High glucose levels, as well as its glucose metabolites, can affect vascular cell function directly via various signaling pathways (Evcimen & King, 2007). The DAG-PKC signaling pathway is a widely studied pathway indicating how diabetes may induce vascular complications. It is believed that hyperglycemia alters signaling pathways, causing cellular dysfunctions and damage (Geralds & King, 2010). Activation of the DAG-PKC pathway, which enhances polyol pathways, leads to increased oxidative stress, and causes overproduction of advanced glycation end products, has been proposed as a mechanism by which hyperglycemia induced diabetic vascular complications, such as DN, may arise (Noh & King, 2007) (Figure 2).

For instance, previous research demonstrated that PKCα-deficient mice were protected against albuminuria, suggesting that PKCα activation is linked to DN (Menne, 2006). Although it is known that PKCα activation is correlated to vascular complications in diabetic patients, the mechanisms are not fully understood.

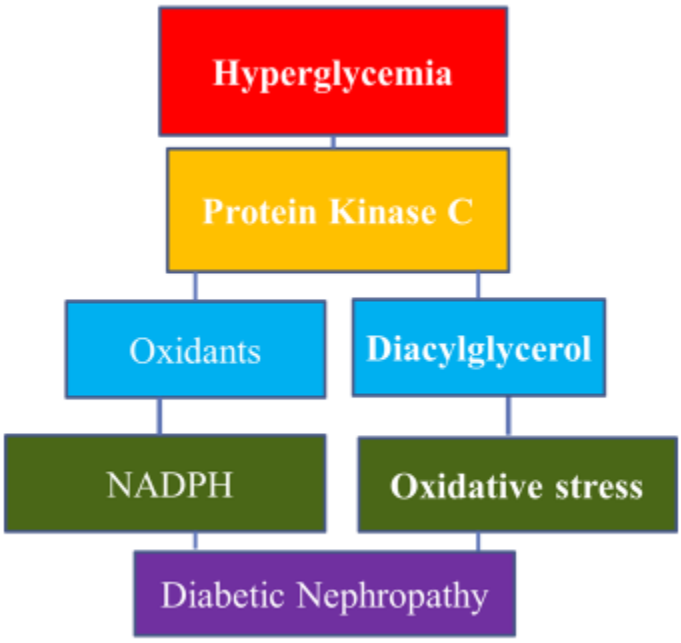


*Figure 2.* Schematic representation of various biological targets of PKC activation leading to diabetic nephropathy (adapted from Geralds, 2010).

**2.3 Protein Kinases**

Protein kinases comprise a sophisticated family of enzymes that regulate the function of nearly all cellular functions. Eukaryotic protein kinases modify other proteins by catalyzing the transfer of a phosphate group to Ser, Thr or Tyr residues located within their protein substrate. The human genome contains 518 protein kinase genes, constituting approximately 2% of all

human genes (Manning, 2002). Up to 30 % of all human proteins are believed to be modified by protein kinases (Manning, 2002). As a consequence, protein kinases have rapidly become major therapeutic targets.

One of the most well studied kinase family members, PKCα, mediates a variety of physiological responses within a cell. PKC family members are phospholipid-dependent serine/threonine kinases that play key roles in signaling pathways involved in regulating cellular growth, proliferation, differentiation and cell death (Freeley, Kelleher & Long, 2011). PKC function, like most protein kinases, is influenced by phosphorylation, which has a central role in its structure and function. In particular, three conserved phosphorylation sites located in the activation loop, the turn motif, and the hydrophobic-motif are important in controlling the catalytic activity, stability and intracellular localization of the enzymes (Freeley, Kelleher & Long, 2011).

PKC family members, like all other eukaryotic protein kinases, are characterized by an N-terminal lobe consisting almost entirely of β-sheets and a C-terminal lobe composed primarily of α-helices (Figure 3) (Taylor & Kornev, 2011). The kinase active site, which is involved in both ATP and substrate binding, lies at the cleft formed between the N- and C-terminal lobes. The N-terminal regulatory domain has two main functions. First, it contains a pseudosubstrate sequence that binds to the substrate-binding cavity and blocks catalytic activity. Binding of the pseudosubstrate region is modulated by several regulatory elements located in the N-terminal lobe, including the C1 domain (involved in DAG binding), the C2 domain (involved in $Ca^{2+}$ binding), and, in the case of atypical PKC (aPKC) family members, the PB1 domain (involved in protein-protein interactions and phosphatidylserine binding) (Yang, 2012; Freely, 2011). These regulatory domains are also useful in distinguishing between different PKC subfamilies.

Currently, nine PKC isoforms have been cloned and characterized. The nine PKC isoforms are classified into three subgroups based on the presence or absence of functional C1 and C2 domains (Figure 3). For example, the conventional PKCs (cPKCs), which are comprised of PKCα, β1, β2 and γ, contain both a functional C1 domain and a functional C2 domain. Meanwhile, the novel PKCs (nPKCs), which consist of PKC δ, ε, η and θ, contain a functional C1 domain but lack a functional C2 domain, thereby rendering them insensitive to $Ca^{2+}$. Finally, the aPKCs, which are comprised of PKC ζ and ι/λ, contain neither a functional C1 domain nor a functional C2 domain. Instead, aPKC family members contain a PB1 domain. These N-terminal regulatory elements are largely involved in regulating the subcellular distribution of PKC family members. For instance, interactions between the C1 domain and the lipid second messenger, DAG, promote the redistribution of cPKC and nPKC family members from the cytoplasm to the plasma membrane. In addition, conformational changes induced by the regulatory elements releases the pseudosubstrate, allowing subsequent binding and phosphorylation of the substrate (Freely, 2011).

The C-terminal lobe is also critical to PKC function. For instance, the C-terminal lobe contains the C3 and C4 domains, which provide critical contacts with ATP and the substrate, respectively, in the active site binding cleft (Nakashima, 2002). Moreover, phosphorylation sites located on the C-terminal activation loop, the turn motif, and hydrophobic-motif are important to PKC function. Many of these phosphorylation sites are conserved in other AGC family members, including the canonical ABC kinases (PKA, PKB/Akt, PKC). Importantly, a non-conserved hinge domain found in PKC family members, known as the V3 region, is present between the N- and C-terminal lobes. When PKC is active, this V3 “hinge” domain may be susceptible to proteolytic enzymes.

This study focuses on PKCα. PKCα is a serine/threonine kinase and a member of the conventional (classical) PKCs (cPKCs), which have four conserved (C1 to C4) regions. This PKC isotype is activated in response to many different kinds of stimuli and translocates from the cytosol to distinct subcellular compartments (e.g., nucleus, sites of focal adhesion, caveolae, etc.) where it acts to phosphorylate its downstream effectors. Therefore, PKCα has been implicated in a variety of cellular functions, including proliferation, apoptosis, differentiation, motility, and inflammation. However, the responses induced by activation or overexpression of PKCα vary depending on the cell type as well as cellular conditions. For example, in MCF-7 human breast cancer cells and the human glioma U87 cell line, PKCα has been implicated in cell growth (Nakashima, 2002). In contrast, it may play a role in cell cycle arrest and differentiation in other types of cells, such as melanoma and lens epithelial cells (Nakashima, 2002). Therefore, alterations of cell responses induced by PKCα are not an intrinsic property of this isoform. Rather, the responses are modulated by dynamic interactions with cell-type specific factors: substrates, modulators and anchoring proteins.

**2.4 Meprins**

Meprins are multidomain zinc metalloproteinases of the astacin family that are highly expressed at the brush border membranes (BBM) of proximal kidneys tubules and small intestines (Norman, 2003). Meprins are also expressed in leukcocytes (Sun, 2009) and certain cancer cells (Norman, 2003). Meprins have been implicated in inflammation as well as cancer growth and metastasis. They are the only members of the astacin family that can be both secreted and membrane bound (Sterchi, 2008). In humans, two meprin genes exist, meprin α and β, that are located on chromosome 6 and 18, respectively. Meprin α and β are evolutionarily related, with their gene products exhibiting 42 % identity at the amino acid level and sharing the same

domain structure (Norman, 2003). The gene products of meprin α and β associate to form either homo- or hetero-oligomers with a multi-domain structure that forms covalent and non-covalent dimers and/or tetramers. For instance, Meprin A consists of a homodimer of meprin α subunits or heterooligomer of α-and β subunits while meprin B is a meprin β homo-oligomer. Importantly, while meprin B and the heteromeric isoform meprin A (α/β) remains membrane-associated due to an integral membrane domain, meprin A (α/α) is secreted (Bond, 2005).

### 2.4.1 Meprin substrates

Meprins are capable of hydrolyzing many substrates *in vitro*, including extracellular matrix (ECM) proteins (e.g., collagen type IV, laminin, fibronectin and nidogen), cytokines (e.g., interleukin-1b), hormones (e.g., insulin), and small peptides (e.g., gastrin 17, cerulein, and sCCK 8 of the gastrointestinal tract) (Hahn, 2003; Oneda, 2008). In addition, these proteases also have the ability to cleave cytoskeletal proteins, such as actin and villin, and bioactive peptides, such as TFG-α and angiotensin (Huguenin, 2008; Ongeri, 2011). Although there are common substrates between meprin A and meprin B, the actual sites of cleavage are isoform specific. For example, meprin B preferentially cleaves acidic amino acids in the P1 sites while meprin A prefers small (e.g. serine, alanine) or hydrophobic (e.g. phenylalanine) residues in the P1 sites (Bertenshaw, 2001). Therefore, it has been suggested that meprins are involved in the modulation of the activity of important extracellular proteins and signaling peptides (Hahn, 2003). Recently, we demonstrated that the catalytic subunit of protein kinase A (PKA) is cleaved by the metalloproteinase Meprin B at its C-terminus, causing an ~20-fold decrease in kinase activity (Niyitegeka, in press, AJP-Renal). The site that is cleaved by Meprin B is highly conserved among various protein kinases, including several PKC family members. We believe that PKC may also be cleaved at the conserved site by meprin B. In addition, we and others have

demonstrated that meprin-mediated cleavage of PKA, alters its kinase activity, suggesting potential functional consequences.

### 2.4.2 Meprins and diabetic nephropathy

Merpin proteinases have the potential to be detrimental at the site of tissue damage, such as in acute renal injury. Linkage analysis has implicated meprin β as a candidate gene for DN in the Pima Indian population, in which 92% of ESRD was attributed to DN (Norman, 2003). Moreover, the meprin β locus on chromosome 18 has been linked to a greater risk of DN in patients with type 2 diabetes (Mathew, 2005).

The two meprin isoforms are deleterious in renal tissue, which indicates a contribution to renal injury. This proteolytic activity of meprins in renal injury indicates multiple functions in health and disease; however, the specific mechanisms underlying these functions are not well understood. The role of metalloproteinases in basement membrane remodeling in particular has recently received a lot of attention due to their ability to influence the progression of renal dysfunction (Oneda, 2008).

In transfected cells, intracellular proteolytic removal of the membrane anchor results in the secretion of the meprin α subunit. In human meprin, its subunit is converted into a secretable form, unlike mouse and rat meprin (Hahn, 2003). Previous research demonstrated that human meprin is phosphorylated following treatment with phorbol 12-myristate-13-acetate (PMA), a PKC-activating phorbol ester. Interestingly, the site of phosphorylation, Ser687, lies within a PKC consensus sequence located in the cytosolic domain of the protein (Hahn, 2003).

# CHAPTER 3

# Methodology

## 3.1 Reagents

Monoclonal mouse anti-PKCα and anti-PKCβe antibodies were purchased from BD Biosciences (Franklin lakes, NJ). Dulbecco's modified Eagle media (DMEM) was purchased from Gibco (Grand Island, NY). Beta-mercaptoethanol, glycerol, Tris base, sodium chloride, 2-[4-(2-hydroxyethyl)piperazin-1yl]ethanesulfonic acid (HEPES) and ethylenediaminetetraacetic acid (EDTA) were purchased from Amresco, Inc. Yeast nitrogen base without ammonium sulfate and without amino acids, galactose, ethanol, glacial acetic acid, sodium orthovanadate, ethyl alcohol (200 proof) and Tween 20 were purchased from Fisher Chemical (Pittsburgh, PA). SC-URA power mix was purchased from MP Biomedicals (Solon, OH). Tetramethylethylenediamine (TEMED), sodium citrate, and sodium dodecyl sulfate were purchased from Sigma-Aldrich (St Louis, MO). The following chemicals were purchased from Bio-Rad (Hercules, CA): blocking grade milk, silver stain plus kit, 40% acrylamide solution, EZRun pre-stained protein ladder, anti-rabbit IgG .

## 3.2 Meprin Incubations to Determine PKC Cleavage/Degradation

To determine whether PKC is cleaved/degraded by meprins, cytosolic-enriched cell lysates from meprin αβ double knockout mouse kidney tissue and cell lysates from Mardin-Darby canine kidney cells (MDCK) cells were incubated with either active, recombinant human Meprin A (α-α) or recombinant rat Meprin B (β-β) for 0-4 hours. 20 μL aliquots were removed from the reaction at 0, 0.5, 1, 2, 3, and 4h. The reaction was quenched by adding 4 μL of 6x SDS-loading buffer. Each sample was then loaded into a 15-well 1.5mm 10 % SDS-polyacrylamide gels, using 20 μl for each lane. Western blot analysis was conducted to quantify

the levels of PKCα present, as described below. In addition to cell lysates, recombinant human PKCα, purified from yeast by GST-affinity chromatography, was incubated with Meprin B for 4 h and analyzed by western blot.

**3.3 Western Blot Analysis**

Western blot analysis was used to assess cleavage and to quantify the levels of PKCα in lysates from Mardin-Darby canine kidney cells (MDCK) and cytosolic-enriched αβ double knockout mouse kidney proteins incubated with meprins. Following incubations, proteins were separated by electrophoresis on a 10 % acrylamide gel and transferred to a nitrocellulose membrane. Non-specific binding sites were blocked by incubation for 1 h at room temperature in 5-8% fat-free milk in Tris-buffered saline with 0.05% Tween-20 (TBS-T). To determine the levels of PKCα and PKCβ expressed in MDCK and kidney proteins, membranes were incubated with mouse monoclonal anti-PKCα and anti-PKCβ antibodies (BD biosciences), respectively, diluted 1:1000 in TBS-T, in TBS with 1 % bovine serum at RT for 1h or at 4$^{o}$ C overnight. Anti-mouse IgG-HRP conjugate (Thermos Scientific), diluted 1:10,000 in TBS-T, was used as secondary antibodies. Bands were detected by chemiluminescence after adding ECL substrate to the membranes and developed on X-ray film.

**3.4 Cell Culture and Protein Extraction for HEK 293 cells**

Cell culture was used to grow non-transfected, meprin α-transfectedand, meprin β-transfected human embryonic kidney cells (HEK 293). Dubelcco's modified Eagle media (DMEM) with 4.5g/L glucose supplemented with 10 % Fetal Bovine Serum (FBS) was warmed in a water bath for 30 minutes at 37 °C. After media was warmed, 10 ml of DMEM media was added to each 10 $cm^2$ cell culture dish. Cells were incubated in a $CO_2$ incubator set at 37° C, 5%

$CO_2$ and 85% humidity until they reached 70-90 % confluence. The media was then changed to DMEM media supplemented with 2.5 % FBS and the cells cultured overnight.

**3.5 Protein Extraction**

Total proteins were extracted from HEK 293 and MDCK cells. The 100 $cm^2$ cell culture dishes containing the cells were rinsed with ice-cold sterile phosphate buffered saline (PBS) three times. 500 µl of radioimmunoprecipitation assay (RIPA) buffer (1M Hepes,1M NaCl,10% Triton-X-100, 10% SDS,100 mM $Na_3OV_4$, 0.5M EDTA and 100X protease inhibitor cocktail) was added to each dish to extract total protein and incubated on ice for 30 minutes. Cells were harvested using a cell scraper and transferred to 1.5 ml centrifuge tubes. Cells were centrifuged for 30 minutes at 21,000 x *g* at 4 °C. Finally, supernatants, containing the cell lysate were transferred to new centrifuge tubes for western blot analysis.

**3.6 Purification of Recombinant PKC Proteins**

Human PKCα containing an N-terminal GST affinity tag was purified from yeast using GST-affinity chromotography. Accordingly, a single colony of yeast transformed with a yeast expression vector encoding GST-PKCα was used to innoculate 5 mL of SC-URA(-) culture media supplemented with 20 % (w/v) glucose and grown overnight (approximately 16 hours until saturation). The following evening, 100 µl of the overnight culture was used to inoculate 50 mL of SCR-URA(-) supplemented with 20 % (w/v) raffinose for approximately 16 hours at 30 °C in an incubator shaker (200 rpm). Once the culture reached an optical density between 0.6 and 1.0 $OD_{600}$, PKC expression was induced for six hours by the addition of 40 % (w/v) galactose to a final concentration of 2 % (w/v). Time points were taken every 2 hours and used to assess induction. Following induction, cells were pelleted for 3 minutes at 850 x g in 50 ml conical tube, washed with $dH_2O$ and stored at -80 º C until purification. To purify GST-PKCα,

cells were lysed by mechanical lysis and purified by GST-affinity chromatography, as described (Newman, 2013). Following purification, the purity of GST-PKCα was assessed by silver staining.

**3.7 SDS-PAGE and Silver Staining**

Purified GST-PKCα was incubated in the presence or absence of recombinant purified Meprin B for 4 h. Aliquots (20 μL each) were removed at the indicated time points and the reaction was quenched by the addition of 4 μL of 6x SDS-loading buffer. Samples were then loaded onto a 10 % SDS-PAGE gel and resolved at 200 volts for ~45 minutes. To assess the extent of cleavage, the bands were visualized by silver stain analysis according to the manufacturer's protocol. Briefly, the acrylamide gel was placed in fixative enhancer solution for 20 minutes before being rinsed in deionized distilled water for 2 x 10 minutes. Samples were stained with development accelerator solution until desired staining intensity was reached. To stop staining, the gel was placed in 5 % acetic acid stop solution for ~15 minutes.

**3.8 Glucose Treatment of Human Embryonic Kidney (HEK) 293 cells**

We used HEK293 cells transfected with cDNA for meprin α and meprin β to determine the impact of meprins on PLC expression following exposure to high glucose. Non-transfected cells were used as controls. Three glucose concentrations were used for each genotype of cells; no glucose (0 mM), low glucose (5 mM), and high glucose (25 mM). The cells were first cultured in DMEM supplemented with 10% FBS and antibiotic/antimycotics in a $CO_2$ incubator at 37 ºC and 5 % $CO_2$ to 50 % confluence. The culture media was then changed to a reduced serum media (2.5 % FBS). Glucose dissolved in PBS was then added to a final concentration of 5 mM or 25 mM. An equivalent volume of PBS was added to the 0 mM, no glucose control. The cells were cultured for an additional 48 hours. Protein lysates were extracted as described in

section 3.4 above. Proteins were separated by gel electrophoresis and analyzed using Western blot analysis with anti-PKCα specific antibodies. The protein levels were quantified by optic densitometry using Bio-Rad's GS800 scanner. The intensities for tubulin were used as a loading control.

**3.9 Immunofluorescence Analysis of Kidney Tissue for PKC and Meprins**

We used immunofluorescence (IF) to analyze paraffin-embedded kidney tissues from wild-type mice with STZ-induced type 1 diabetes. For the deparaffinization step slides, each slide was placed in xylene for 5 minutes three times. Next, slides were placed in 100% ethanol for 10 minutes two times. Slides were placed in 95 % ethanol for ten minutes two times. Slides were placed in deionized distilled water for 5 minutes 2 times. Antigen unmasking step slides were brought to a boil in 10 mM sodium citrate, pH 6.0, for 10 minutes. Slides were cooled for 30 minutes and rinsed in 10x PBS. Immunostaining slides were blocked in blocking buffer (1X PBS , normal goat serum, and Triton X-100) for 60 minutes in a humidity chamber. Next, slides were incubated with primary mouse monoclonal anti-PKCα antibodies (diluted1:100), rabbit polyclonal anti-meprin B (HMC77) (diluted 1:400) or rabbit polyclonal anti meprin A antibodies (HMC14) diluted 1:200) overnight. Slides were rinsed 3 times in 1x PBS for 5 minutes each. Slides were incubated with fluorophore-conjugated secondary antibodies rabbit Alexfluor488 (1:1000 dilution) and/ or mouse Alexafluor555 (1:1000 dilution) with DAPI for 1 hour at room temperature in the dark. Slides were rinsed three times in 1x PBS for 5 minutes each. Prolong® Gold Anti-Fade Reagent was added to slides before coverslips were mounted and left to dry overnight at room temperature.

# CHAPTER 4

## Results

### 4.1 Meprin B Degrades PKCα Present in Kidney Proteins and Kidney Cell Lysates.

To determine whether meprins cleave PKCα present in cells derived from native tissue, kidney tissue lysates from αβ-knockout (KO) mice were incubated with activated forms of purified meprin A and meprin B, and PKCα levels were assessed by Western blot analysis (Figure 3). While no change in PKCα levels was observed after a 4 h incubation with meprin A, a marked reduction in PKCα levels was observed after only 0.5 h incubation with meprin B, with PKCα levels decreasing below the detection limit of the assay after 2 h. These results suggest that PKCα is degraded by the meprin B isoform in a time-dependent manner.

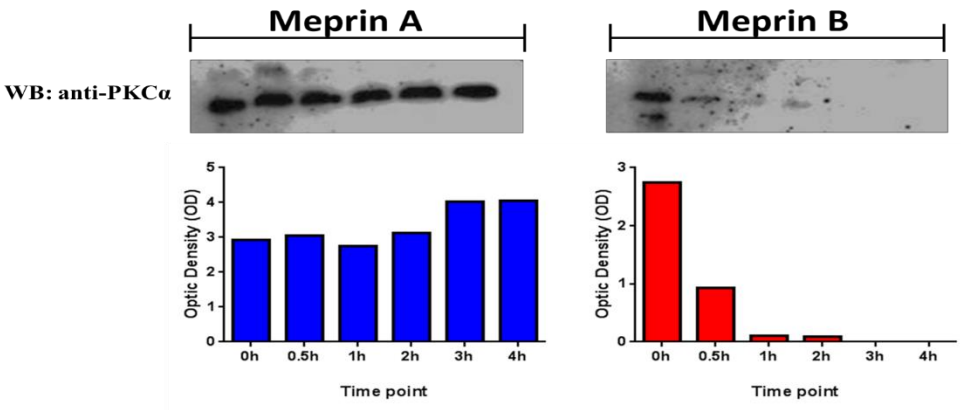


*Figure 3.* Meprin degradation of *PKCα* present in kidney proteins from meprin αβ double knockout mice.

The same outcome was observed when MDCK cell lysates (which do not normally express meprins) were incubated with meprin A and meprin B and PKCα levels were analyzed at various times over the course of the reactions (Figure 4). While PKCα levels did not change following incubation with Meprin A, PKCα levels decreased dramatically after 0.5 h of

incubation with Meprin B. The levels of PKCα continued to decrease in a time-dependent manner at the 1 h and 2 h time points until no PKCα could be observed after 3 h. Together, these data suggest that PKCα is preferentially cleaved by meprin B in a time dependent manner.

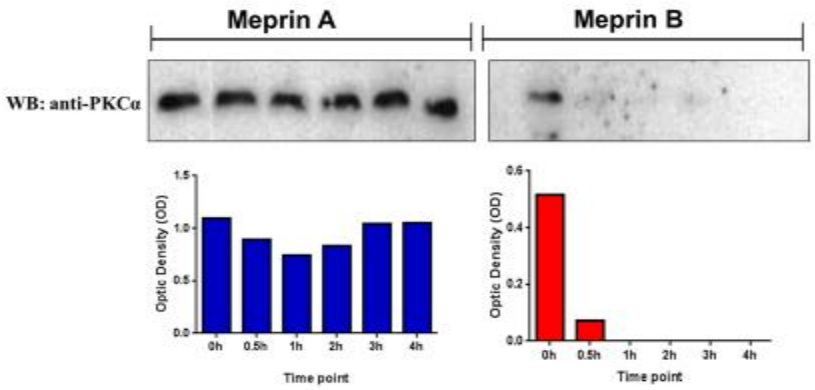


*Figure 4.* PKCα levels in MDCK cell lysates incubated with active meprin A and meprin B.

In order to confirm that PKCα is degraded by meprin B, recombinant PKCα was purified by GST-affinity chromatography, incubated with active meprin B for 3 h, and the products analyzed by Western blot (Figure 5). In the absence of meprin B, recombinant PKCα migrated as a doublet, presumably corresponding to the phosphorylated (active) and unphosphorylated forms of the protein. Interestingly, only the upper band decreased following incubation with meprin B, raising the possibility that meprin B preferentially cleaves the phosphorylated form of recombinant PKCα. Further studies are currently underway to test this hypothesis.

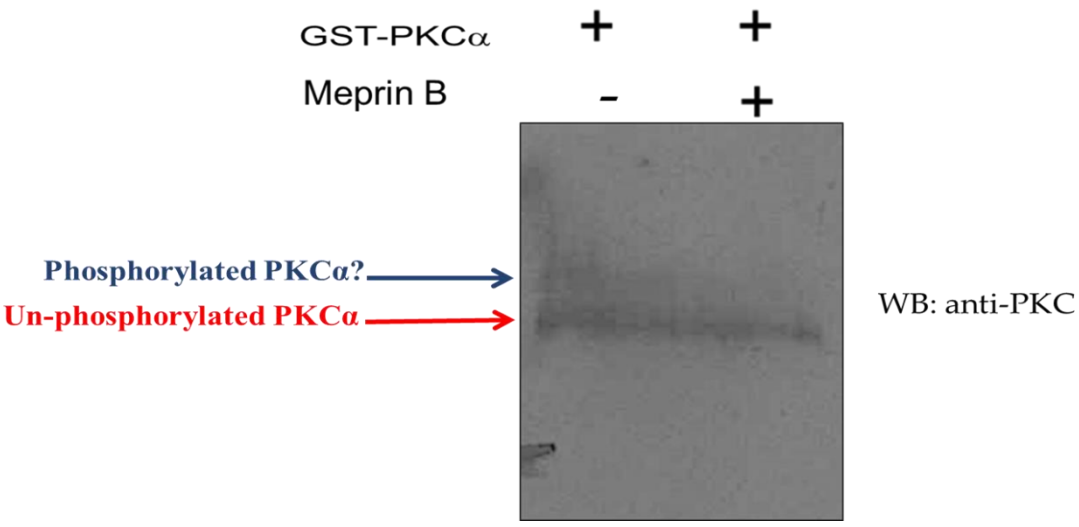


*Figure 5.* Immunoblot of PKCα following incubation of purified recombinant PKCα with activated purified meprin B. In order to confirm that degradation of PKCα is meprin B-specific, recombinant PKCα was purified by GST-affinity chromatography, incubated with active meprin B for 3 h, and the products analyzed by western blot (unphosphorylated and a putatively phosphorylated species are indicated).

**4.2 Impact of Glucose Treatment on PKCα Expression in HEK 293 Cells**

To determine the impact of meprin expression on PKCα in cells exposed to a high glucose environment, either untransfected, *meprin α*-transfected or *meprin β*-transfected HEK293 cells were treated with 0 mM (no glucose control), 5 mM (low glucose) or 25 mM (high glucose) (Figure 6). Western blot analysis was used to assess the levels of PKCα. Untransfected cells appeared to have lower relative PKCα baseline levels than meprin α and meprin β transfected cells.

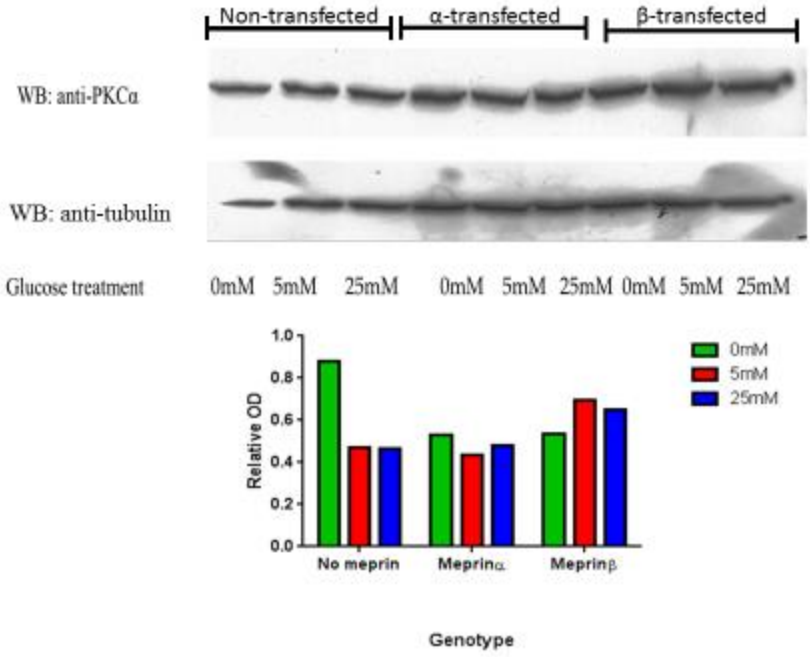


*Figure 6.* Effect of glucose treatment on PKCα levels in HEK 293 cells. To determine the impact of meprin-mediated cleavage of PKCα in cells exposed to a high glucose environment, either untransfected, meprin α-transfected or meprin β-transfected HEK293 cells were treated with 0 mM (no glucose control), 5 mM (low glucose) or 25 mM (high glucose). Western blot analysis was used to assess the levels of PKCα. The graph above shows the optical density of anti-tubulin and anti-PKCα blots. Untransfected cells appeared to have lower band intensity than meprin α- and meprin β-transfected cells.

### 4.3 Impact of Diabetes on PKCα Levels In Vivo

Recent studies suggest that meprins are not only activated at the plasma membrane in models of diabetes and diabetic nephropathy, but that they are also redistributed to the cytosol where they act on cytosolic proteins (Bylander, 2008; Ongeri, 2011). Therefore, it is possible that meprins can act on PKCα *in vivo* under conditions of chronic or acute kidney damage. To examine this question further, immunofluorescence was employed to determine the distribution

of meprins and PKCα in kidney tissues derived from non-diabetic and diabetic mice. In the non-diabetic control and diabetic kidney tissues, meprin A is only seen in the brush border membrane of proximal kidney tubules (Figure 7). In comparison to the non-diabetic control, the diabetic mouse kidney tissues showed meprin A in the glomerulus (Figure 8).

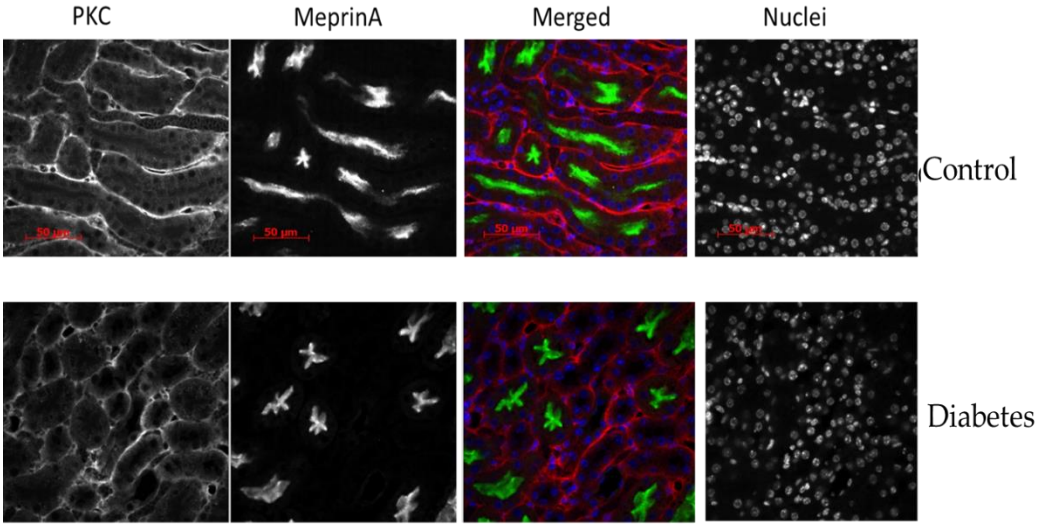


*Figure 7.* Immunofluorescence images of control (top) and STZ-treated (bottom) kidney tissue for PKCα and meprin A in kidney tubules (green: meprin A; red: PKCα; blue: DAPI-stained nuclei).

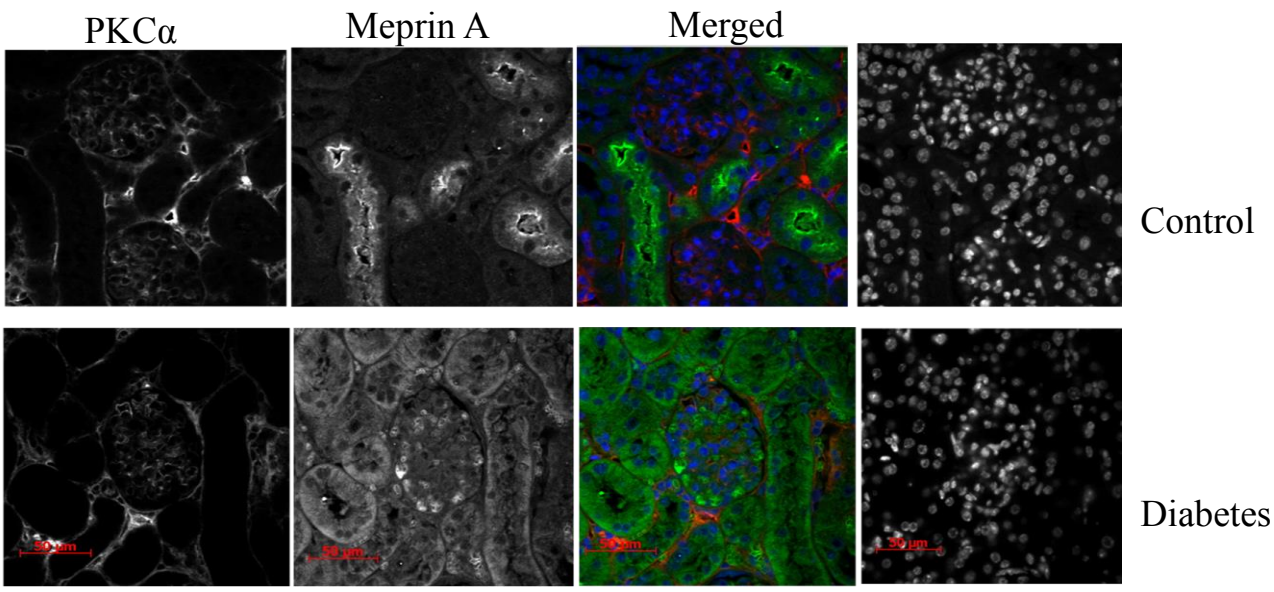


*Figure 8.* Immunofluorescence analysis of control (top) and STZ-treated (bottom) kidney tissue for PKCα and meprin A in kidney glomeruli (green: meprin A; red: PKCα; blue: DAPI-stained nuclei).

# CHAPTER 5

## Discussion and Future Research

Diabetic nephropathy is the leading cause of ESRD with high mortality and morbidity rates (Reeves & Andreoli, 2000). In the U.S., DN accounts for about 40 % of new cases of ESRD (Molitch, 2008). Complications of DN include a set of structural and functional abnormalities within the kidney, such as cellular hypertrophy, an increase in the thickness of the glomerular basement membrane (GBM) and the tubular basement membrane (TBM), tubulointerstitial fibrosis, and mesangial cell expansion with extracellular matrix, also known as glomerulosclerosis (Wada & Makino, 2013). Meprins, which are metalloproteinases that are abundantly expressed at the BBM of kidney tubules, are known to degrade ECM proteins. Meprins are also expressed in podocytes, skin, and leukocytes. Inappropriate activation of protein kinase C (PKC) is known to have implications in DN progression. To examine the role of meprins in regulating intracellular PKCα activity, MDCK lysates and αβ-knockout kidney proteins were used to determine whether meprins cleave/degrade PKCα. Results from this study suggest that PKCα is cleaved by meprin B, but not meprin A. Our data indicate that PKCα degradation is specific to meprin B and occurs in a time dependent manner in both MDCK cell lysates and knockout kidney proteins. To further investigate whether cleavage of PKCα is meprin B-specific, recombinant PKCα was purified and incubated with activated meprin B. The phosphorylated form of PKCα seemed to be preferably cleaved by meprin B. Since PKCα is known to play a role in the pathogenesis of DN, knowing that it is a target for meprin B may provide a better understanding of the PKC pathway involvement in DN, and mechanisms by which meprins modulate kidney injury in DN. To access the impact of high glucose on PKCα

expression in the presence of meprins, HEK 293 cells were treated with no, low and high glucose. There was not a major difference in PKCα expression between meprin α- and β-transfected cells. In comparison to non-transfected HEK 293 cells, the meprin-transfected cells appeared to have higher PKCα expression. This may indicate PKCα expression is being regulated by meprins under the influence of glucose. Additional experiments are needed to verify this claim. Finally the impact of diabetes on PKCα in the presence meprins was assessed using immunofluorescence analysis. The data show that PKCα is expressed in both tubular cells and cells in the glomeruli. While meprin expression is predominantly in the BBM of proximal tubules, we observed glomeruli expression of meprin A in diabetic mice. Meprin B may be an important factor in the PKC pathway, which may have important implications because meprins are known to have detrimental effects on kidney tissues (Bond, 2005). The regulation of PKC in DN is not fully understood so overall results may indicate that PKCα is modulated by meprin B. Future studies need to be done to validate these claims.

Though we have determined that PKCα is a substrate of meprin B, it is still not clear where on PKCα meprin B is cleaving. Therefore, mass spectrometry analysis will be performed to further evaluate the specific site(s) of meprin B cleavage in PKCα. Likewise, it will be important to understand the impact of meprin B cleavage on PKCα activity and/or substrate selection. For the former, the activity of PKCα before and after meprin B-mediated cleavage will be determined using a model PKC substrate (e.g., the peptide substrate, PKCtide) while functional protein microarrays composed of ~19,000 unique human proteins can be used to assess the latter. Finally, though we have demonstrated that cleavage of PKCα is meprin isoform specific with respect to the meprin isoforms, meprin A and B, in the future it will be interesting to determine whether meprin degradation is isoform specific with respect to other PKC family

members (e.g., PKC β1 and β2). Based on previous studies with the catalytic subunit of PKA, it is hypothesized that meprin B will affect PKC function by reducing or abolishing its activity.

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