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Meprin Interaction With Osteosarcoma 9 (Os9) And The Hypoxia Response Gene, Hypoxia Inducible Factor 1 (Hif1) Alpha, In Kidney Tubular Cells

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Biographical Sketch

Barry L. Martin is a native of North Carolina living in the Piedmont region of the state. Barry attended Southern Alamance High School where he was very active in extracurricular activities including serving as an officer of student council, and as a local, district, and state officer in 4-H. Barry has received numerous recognitions for his leadership and dedication from local vicinity as well as from the State. Barry attended Lenoir-Rhyne College in Hickory, NC and received his B.A. degree in French. While at Lenoir-Rhyne, Barry enjoyed being a member of the historic A Cappella Choir for all four years. As a child, Barry always had a passion for science. After graduating, he began working full-time as a laboratory technologist at Carolina Biological Supply Company where he had been working part-time since the day after graduating from high school.

Barry went back to school at the University of North Carolina at Greensboro and completed his B.S. degree in biology. After almost 10 years at Carolina Biological, Barry decided that it was time to leave to explore new opportunities in life. After a few years working in the service industry, Barry returned to his roots gaining employment as a reference microbiologist for a major laboratory. Barry decided to become board certified as a medical technologist. In order to receive certification, Barry obtained his clinical laboratory science degree from Winston-Salem State University graduating magna cum laude. Barry is board-certified as a medical laboratory scientist by the American Society of Clinical Pathology.

To advance his career, Barry enrolled in the Master of Science degree in Biology program at North Carolina A&T State University in the fall of 2011. After graduation, Barry plans to continue to work in the field of medical laboratory science.

Barry was brought up in Lutheran Church where he is a life-long member of St. Paul's Lutheran Church in Burlington, N.C. Barry has one younger brother named Jeffrey. His parents (Cletus and Marcia), sibling, and multiple immediate family members reside in the Snow Camp/Burlington area. In his spare time, Barry enjoys traveling, learning about different cultures, and reading classical novels.

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Abbreviations and Symbol Key

µl	microliter
µM	micromole or micromolar
Ala	alanine
APS	ammonium persulfate
Asp	aspartic acid
ATCC	American Type Culture Collection
ATP	adenosine-5´-triphosphate
BBM	brush-border membrane
bp	base pair
BSA	bovine serum albumin
C	celsius
$CaCl_2$	calcium chloride
cDNA	complementary deoxyribonucleic acid
cm	centimeter
CO_2	carbon dioxide
dd	distilled deionized
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
ERAD	ER-associated degradation pathway
ESRD	end stage renal disease
FBS	fetal bovine serum
x g	relative centrifugal force
g	gram
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Glu	glutamic acid
H_2O	water
HEK293	human embryonic kidney 293 cells
Hepes	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HIF	hypoxia-inducible factor
HIF-1	hypoxia inducible factor 1
hr	hour
IgG	immunoglobulin G
IR	ischemia reperfusion
kB	kilobase
KCl	potassium chloride
L	liter
Leu	leucine
M	molar

MDCK	Madin-Darby Canine Kidney
MEM	minimum essential medium
$MgCl_2$	magnesium chloride
min	minute
ml	milliliter
mM	millimole or millimolar
mRNA	messenger ribonucleic acid
Na_3VO_4	sodium orthovanadate
NaCl	sodium chloride
OS9	osteosarcoma 9
P1´,P2´	specific amino acid position flanking the catalytic site of an enzyme
PBS	phosphate-buffered saline
PHD	prolyl hydroxylase domain protein
Pro	proline
pVHL	von-Hippel-Lindau protein
qs	quantum satis
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
SDS	sodium dodecyl sulfate
TBS	tris-buffered saline
TBS-T	tris-buffered saline with Tween-20
TEMED	tetramethylethylenediamine
Thr	threonine
Tris	trisaminomethane
V	volts
VIS	visible light
X	times
α	Greek letter alpha
β	Greek letter beta

Abstract

Meprins, metalloproteases that are highly expressed in the brush-border membranes of kidney proximal tubules, have been associated with ischemia-reperfusion induced kidney injury. Osteosarcoma 9 (OS9), a peripheral membrane protein, has been shown to selectively interact with the carboxyl-terminal tail of meprin beta (β). Additionally, OS9 was shown to promote oxygen-dependent degradation of the hypoxia-inducible factor 1α (HIF1α), a protein involved in oxygen homeostasis. However, it is not known if OS9 is a meprin substrate. The objective of this study was to determine if OS9 is a meprin substrate, and whether there is a correlation between expression of meprins, OS9, and HIF1α under hypoxic conditions.

To determine if meprins are capable of degrading OS9, purified human OS9 was co-incubated with activated recombinant forms of meprin A and B. Our results show that meprin B cleaves/degradation OS9. This was not observed for meprin A, suggesting isoform-specific cleavage. Mardin-Darby canine kidney (MDCK) cells and human embryonic kidney 293 cells transfected with meprin α or β cDNA were depleted of oxygen by exposure to 125 μM cobalt chloride. Non-transfected cells served as controls. Western blot analysis was used to evaluate the nuclear fraction levels of OS9 and HIF1α. Our data showed that HIF-1α stabilized in the nucleus in a time-dependent manner and the levels of OS9 increased in non-transfected controls cells depleted of oxygen. To our knowledge, this is the first study to identify OS9 as a meprin B substrate. The degradation of OS9 by meprin B could impact the hypoxic response in kidney cells.

CHAPTER 1

Introduction

1.1 Life's Requirement for Oxygen and Energy

Cells utilize oxygen for energy production in the form of adenosine-5´-triphosphate (ATP) to carry out cellular functions. During oxidative phosphorylation, oxygen serves as an electron acceptor in the formation of ATP. An adequate supply of oxygen is essential for cells to perform their functions and for cells to survive. Cells must try to compensate under low levels of oxygen known as hypoxia. If a cell is unable to compensate, or if there is an abrupt lack of oxygen, then it can lead to cell death. *In vitro* models have been developed and to study the effects of hypoxia and ATP-depletion in cells. These methods include using pharmacological agents to induce hypoxia and ATP-depletion in cultured.

1.2 Ischemia-Reperfusion Injury

An organ may experience ischemia (restriction of blood supply) by an event such as physical blockage, organ transplantation, or diabetic chronic kidney failure. The organ is starved of oxygen and nutrients until blood flow restoration occur. The sudden rush of blood, containing oxygen and other nutrients, creates a catastrophic event within the organ resulting in tissue damage or disease. The damage resulting from the return of blood after a period of ischemia is termed ischemia-reperfusion injury. Ischemia results in oxygen- and ATP-depletion both of which have been shown to disrupt the cell cytoskeleton and its associated membrane structures (Ongeri, Anyanwu, Reeves, & Bond, 2011). The damage caused to cells under hypoxia and the inability to produce ATP is of great significance. Research is required to understand the pathogenesis of disease to organs that have experienced ischemia-reperfusion injury.

1.3 Meprins and Osteosarcoma 9

Meprins are metalloproteases that are highly expressed in the brush-border membranes of kidney proximal tubules. Meprins are capable of degrading extracellular matrix proteins, and processing certain bioactive peptides. Changes in the level of expression and localization of meprins have been associated with the pathology of ischemia-reperfusion induced kidney injury in mice and rats (Bylander et al., 2008; Carmago, Shah, & Walker, 2002; Trachtman, Valderrama, Dietrich, & Bond, 1995; Walker, Kaushal, & Shah, 1998). Recent research has identified two cytoskeletal proteins, actin and villin, as meprin substrates (Ongeri et al., 2011).

Osteosarcoma 9 (OS9) is a protein associated with the cytoplasmic side of the endoplasmic reticulum. OS9 plays a role in the endoplasmic reticulum (ER) associated degradation pathway, and binds to the key regulator of cellular hypoxic response known as hypoxia-inducible factor 1 (HIF1). In 2002, the research of Litovchick et al. showed that OS9 interacts specifically with the intracellular region of meprin B. However, it is not known whether OS9 is a meprin substrate. Beak et al. (2005) demonstrated that OS9 is a negative regulator of HIF-1α. To date, no research has provided a correlation between the expression levels of OS9 and the master regulator of homeostasis gene of HIF1α.

1.4 Objectives and Impact

The objective of the current research was to determine if OS9 is a meprin substrate and investigate the mechanism(s) by which meprins enhance damage to the kidney, using an *in vitro* kidney cell model. The central hypothesis is that OS9 is a substrate of meprin. A second objective was to evaluate how meprins impact the expression of OS9 and the hypoxia response gene HIF-1α.

Meprins have been associated with damage and disease to the kidneys during ischemia reperfusion and diabetic chronic kidney failure. The identification of meprin substrates and its role in the hypoxia response would be useful in the development of treatments for patients undergoing kidney transplants. Stopping this process or reducing the rate of the enzyme would minimize organ damage which occurs in the process of ischemia-reperfusion injury.

CHAPTER 2

Literature Review

2.1 Ischemia-Reperfusion

Periods of time may exist when blood flow is cut off from an organ; thus, oxygen and nutrients can no longer be delivered to the organ. The shortage of blood supply is termed ischemia, and hypoxia occurs during ischemia. Reperfusion is when blood flow is restored after a period of deficient blood flow. Situations which could lead to (ischemia-reperfusion (IR) include immobility, stroke, heart attack, organ transplantation, transfusion, physical blockage, and diabetic chronic kidney failure. The lack of oxygen and nutrients from blood during the ischemic period creates a condition in which the restoration of circulation results in inflammation and oxidative damage through the induction of oxidative stress rather than restoration of normal function. Ischemic injury is the leading cause of acute kidney disease (Gunaratnam & Bonventre, 2009). Five percent of hospital patients are inflicted with ischemic acute tubular necrosis with a mortality rate of 50% (Ympa, Sakr, Reinhart, & Vincent, 2005). Studies show that the pars recta (SC segment) of proximal tubules is the most susceptible to injury (Heyman, Shina, Brezis, & Rosen, 2002; Lieberthal & Nigam, 1998). Chronic hypoxia leads to end stage renal disease. The important effector of tubular cell injury during prolonged hypoxia is ATP-depletion (Devarajan, 2006). Reperfusion injury develops hours or days after the influx event (Kosieradzki & Rowinski, 2008). The sudden rush of flow back into the organ causes a cascade of events. A disruption in the cytoskeleton leads to loss of brush-border, a breakdown of cell junctions, and the incorrect relocalization of sodium-potassium ATPases from the basal surface to the apical surface (Gunaratnam & Bonventre, 2009). Newly freed intracellular molecules of

calcium activate proteases and phospholipases. This reperfusion event is the causative agent of oxidative injury to tubular cells (Bonventre & Zuk, 2004).

Ischemic diseases of the kidney lead to an imbalance of vasoactive substances and vasoconstrictors. The accumulation of vasoconstrictors initiates pro-inflammatory and chemotactic cytokines that begins the up-regulation of adhesion molecules. Leukocytes adhere within the site and release cytotoxic cytokines, reactive oxygen species, and proteolytic enzymes resulting in even more damage to the tubular cells (Bonventre & Zuk, 2004). Cells will attempt to recover from the ischemia-induced damage by repairing/regenerating themselves via undergoing processes of apoptosis, autophagy, and/or necrosis (Kosieradzki & Rowinski, 2008). The extent of damage to the organ depends on whether more cells die or if more cells are able to regenerate.

2.2 Meprins

Meprins are zinc metalloproteases that are highly expressed in the brush-border membranes (BBM) of kidney proximal tubules. Meprins belong to the astacin family (J. S. Bond & Beynon, 1995; Johnson & Bond, 1998). Meprins are also found in the skin, pancreas, testis, regions of the brain, liver, heart, and leukocytes (Becker-Pauly et al., 2007; Crisman, Zhang, Norman, & Bond, 2004; Huguenin et al., 2008; Kenny & Ingram, 1987; Molitoris, Dahl, & Geerdes, 1992). Meprins are capable of degrading extracellular matrix proteins such as collagen and fibronectin (Kaushal, Walker, & Shah, 1994; Walker et al., 1998), and processing bioactive proteins such as neurotension and gastrin (Chestukhin, Muradov, Litovchick, & Shaltiel, 1996; Sterchi, Naim, Lentze, Hauri, & Fransen, 1988). Meprins are the only known endopeptidase in the BBM that degrades proteins: others either only degrade small polypeptides, or are exopeptidases (J. S. Bond, and Jiang, W., 1997). Five percent of the total protein found in BBM

is meprin with expression being localized to the apical membrane of proximal tubule S3 cells (Craig, Reckelhoff, & Bond, 1987). Meprins were identified as the major matrix-degrading enzymes in rat renal tubules (Kaushal et al., 1994; Walker, Kaushal, & Shah, 1994).

The proteolytic enzymatic activity of meprins makes them essential components of cellular activities. Due to their destructive potential, meprins must be highly regulated. Regulation is accomplished through zymogen formation, inhibition, localization to specific compartments, and transcriptional regulation (Bertenshaw, Norcum, & Bond, 2003). Meprins contain a signal peptide that directs the protein to the lumen of the endoplasmic reticulum (ER) during synthesis, and a propeptide that inhibits activity (Johnson & Bond, 1997). The structure of meprins consists of two subunits, α and β (Johnson & Hersh, 1992). The subunits are 42% identical at the amino acid level, highly glycosylated, and form disulfide-linked homo- or heterodimers (J. S. Bond & Beynon, 1995; Marchand, Tang, & Bond, 1994). Each subunit has astacin-like catalytic domain with several protein-interaction domains (Tsukuba & Bond, 1998). Meprin A is a homooligomer of α-subunits or a heterooligomer of α/β subunits, while meprin B is a homooligomer of β-subunits (Johnson & Bond, 1998). Although both forms of meprins prefer substrates containing at least 6 amino acids (Butler & Bond, 1988), meprin A selects amino acids that are small and hydrophobic within the P1´ site and prefers proline residues in P2´ site, while meprin B is predominantly an ASP/Glu-N peptidase with a preference towards acidic residues within the P1´site (Bertenshaw et al., 2001). These differences can account for the diverse functions of the 2 meprin isoforms.

The research of Bertenshaw (2003) provided information on the structure of meprins. Meprin α-subunits associates into larger multimers while meprin B does not form mass complexes of higher levels. Dimers of α/β only form tetramers. The latent form of

homooligomer meprin A forms even larger complexes to the activated form. The degree of multimerization has been deemed to be dependent upon the protein and salt concentration.

The location where meprin is found depends upon the subunit. Meprins containing at least one β-subunit remain membrane-bound due to the transmembrane domain located near the carboxyl terminus of the β-subunit (J. S. Bond & Beynon, 1995). Membrane-bound forms of kidney meprin have been found to be concentrated in the juxtamedullary region of cortex (Craig et al., 1987), and it was determined that meprin β-subunits in the mouse kidney are primary in the latent, proenzyme form (Butler & Bond, 1988). Meprin A is secreted while mouse meprin B and heterooligomer A are membrane-bound. Homooligomer A is able to be freed from the membrane due to a 56 amino acid inserted domain that permits the proteolytic event during maturation (Tang & Bond, 1998).

2.3 Meprins and Ischemia-Reperfusion Induced Renal Injury

One of the functions of the kidney is to provide clearance of plasma polypeptides (Cuber, Bernard, Gibard, & Chayvialle, 1989). Chronic renal failure patients have higher levels of peptides involved in gut mortality. Meprins play a role in the catabolism of extracellular matrix (ECM) proteins. The potential exist where the absence of meprins may account for some of the accumulation of ECM proteins that leads to fibrosis resulting in ESRD (Bertenshaw et al., 2003). Knowledge on the role(s) of meprins in ischemia-reperfusion induced injury is growing. Changes in the level of expression and localization of the meprin β-subunit have been associated with the pathology of ischemia-reperfusion-induced kidney injury in mice and rats (Bylander et al., 2008; Carmago et al., 2002; Trachtman et al., 1995; Walker et al., 1998). It has been proposed that in IR-induced acute renal injury, depletion of oxygen and ATP results in accumulation of intracellular sodium, calcium, and reactive oxygen species. These changes are

believed to activate various enzyme systems including proteases, resulting in disruption of the BBM cytoskeleton and membrane damage, subsequently leading to necrosis and apoptosis (Molitoris, Leiser, & Wagner, 1997). Meprin is cytotoxic, and may play a role in IR injury (Carmago et al., 2002). The latent homooligomer form of meprin A is not a toxic agent in IR (Bylander et al., 2008), suggesting that activation occurs in IR.

Studies with mouse models have increased our knowledge of the influence of meprins in IR-induced renal damage. In 1995, Trachtman el al. showed that non-congenic mice strains with lower renal meprin levels developed less renal injury following IR. In contrast, strains of mice with higher levels of the heterooligomer meprin A (α/β) had more severe IR damage when compared to strains that express meprin B only (Bylander et al., 2008). In meprin B knockout mice there is less disruption and shedding in proximal tubule cell membranes, reduced inflammatory response, and an overall better preservation of kidney function after IR (Bylander et al., 2008). In wild-type mice, kidney meprin B and homooligomeric meprin A are latent due to their prosequences that inhibit proteolytic activity (Butler & Bond, 1988; Villa, Bertenshaw, Bylander, & Bond, 2003). The prosequence is removed, via an unknown mechanism, in membrane-bound meprin A. Bylander et al., (2008) propose that the activated membrane-bound meprin A is the most likely damaging factor *in vivo* after IR.

2.4 Osteosarcoma 9

Research has shown that meprins cause damage to the kidney subjected to ischemia-reperfusion injury. The identity of meprin substrates will help in understanding the mechanisms by which damage is occurring within the kidney. Osteosarcoma 9 has been shown to interact with the C terminal tail of meprin β, and this interaction may provide a mechanism by which meprins modulate the hypoxia response. OS9 plays a role in the Endoplasmic reticulum (ER)-

associated degradation pathway. Proteins that fail to fold or assemble with partnered subunits are selectively removed from the endoplasmic reticulum (ER) via ER-associated degradation pathway (ERAD). Proteins selected for the ERAD pathway are polyubiquitinated and relocated into the cytosol for proteasome degradation (Romisch, 2005). Studies have shown that osteosarcoma amplified 9, endoplasmic reticulum lectin (OS9) are upregulated in response to ER stress, and OS9 is associated with ERAD machinery and ERAD substrates (Alcock & Swanton, 2009). With the use of RNAi, OS9 was proven to be required for efficient ubquitination of glycosylated ERAD substrates. OS9 aided in the transfer of misfolded proteins (Alcock & Swanton, 2009). Osteosarcoma amplified 9, endoplasmic reticulum lectin is a protein coding gene (GenBank ID: 10956) located on 12q13 chromosome of humans that is overly expressed in osteosarcomas (Su, Hutter, Trent, & Meltzer, 1996). OS9 is known to bind to HIF, and promote degradation of one of its subunits (Baek et al., 2005) . The analysis of OS9's cDNA sequence revealed 2785 bp with an open reading frame of 667 amino acids residues (Su et al., 1996). Su et al. determined that the OS9 gene encoded a 2.8 kB mRNA transcribed in all of the tissue samples examined; therefore, OS9 is ubiquitously expressed in human tissues.

2.5 Interaction Between Meprin β and OS9

OS9 is a peripheral membrane protein associated with the cytoplasmic side of the ER, and OS9 may be involved in ER-Golgi transport of meprin B (Litovchick, Friedmann, & Shaltiel, 2002). Meprin A undergoes proteolysis in the ER resulting in the removal of its short carboxyl-terminal cytoplasmic tail (Johnson & Hersh, 1992). Litovchick et al. published work in 2002 (Litovchick et al.) with the purpose of identifying proteins that interact with tail maintained by meprin B, and to deduce the role of the tail. Litovchick earlier in 1998 found that the cytoplasmic domain of rat meprin B is indispensable for its ER-to-Golgi transport, and removal

results in entrapment of meprin B in ER (Litovchick, Chestukhin, & Shaltiel, 1998). Litovchick's research in 2002 demonstrated that OS9 interacts specifically with the intracellular region of meprin B. OS9 is a peripheral membrane protein associated with the cytoplasmic side of the ER, and OS9 may be involved in ER-Golgi transport of meprin B. Non-spliced OS9 binds to the tail of meprin B. The ^{674}Thr-Ala^{689} region of meprin B is critical for the binding of OS9 (Litovchick et al., 2002). This same region is also required for ER-Golgi transport. Litovchick proposes that OS9 interaction with the tail may occur during maturation in ER. In addition, immunofluorescence and fractionation showed that OS9 cell distribution is similar to some ER chaperones.

2.6 Hypoxia Response Genes

A cell survives and thrives under normoxic (normal oxygen) conditions. When the levels of oxygen drop, then the cell is able to sense the difference in the oxygen level. The cell can alter its energy metabolism using anaerobic glycolysis to produce ATP, but this is only a short-term solution. The cell must respond to this environmental stimulus in order to maintain regulation, and for long-termed survival. Hypoxia induces genes to supply oxygen; with hypoxia inducible factor 1 (HIF-1) controlling the expression of most of these genes (Semenza, 1998). A hypoxia inducible factor (HIF) is the cellular response to hypoxia. HIF is the master regulator of hypoxia-response genes (Myllyharju & Schipani, 2010), and HIF-1 functions as a master regulator of oxygen homeostasis that mediates changes in gene transcription in response to changes in cellular oxygen levels (Baek et al., 2005). An alternate means of ATP production is achieved by providing an oxygen-independent mechanism modulated by HIF-1 (Baek et al., 2005). HIF-1 is required for transcriptional activation mediated by the erythropoietin gene enhancer in hypoxic cells (Semenza, 1994). Experimentation performed by Wang (1995)

evaluated the levels of protein and RNA in cells exposed to 1% oxygen for a predetermined time period before oxygen was replenished back to 20%. The RNA and protein levels rapidly decayed which confirms HIF-1's role as a mediator of transcriptional response to hypoxia. The structure of HIF-1 consists of a heterodimer composed of 1 of 3 α-subunits (HIF-1α-2α-or-3α) and a β-subunit (Ema et al., 1997; Gu, Moran, Hogenesch, Wartman, & Bradfield, 1998; Tian, McKnight, & Russell, 1997; Wang & Semenza, 1995). HIF-1β is constitutively located within the nucleus (Berchner-Pfannschmidt et al., 2004). HIF-1 (alpha and beta) are basic-helix-loop-helix-PAS proteins with expression being regulated by cellular oxygen tension (Wang, Jiang, Rue, & Semenza, 1995). The proteolytic stability and transcriptional activity of HIF-1α -and HIF-2α is regulated by two separate oxygen-dependent hydroxylation events (Myllyharju & Schipani, 2010). Under normoxia, little to no HIFα is detectable in the nucleus. HIFα contains an oxygen-depended degradation domain where two –Leu-X-X-Leu-Ala-Pro- sequences are hydroxylated during normal conditions by HIF prolyl 4-hdroxylase (HIF-P4H) (Bruick & McKnight, 2001; Epstein et al., 2001; Ivan et al., 2001). The 4-hydroxyproline residues formed by the HIF-P4Hs are required for the binding to von Hippel-Lindau E3 ubiquitin complex (Myllyharju & Schipani, 2010). This process is known as the ubiquitin proteasome pathway where HIF1α is constitutively synthesized, but destroyed when normoxic conditions exist (Berchner-Pfannschmidt et al., 2004; Kallio, Wilson, O'Brien, Makino, & Poellinger, 1999; Salceda & Caro, 1997). HIFα is degraded by the 26S proteasome (Huang, Gu, Schau, & Bunn, 1998). The dependency of oxygen, during the binding of the von-Hippel-Lindau protein (pVHL) (Maxwell et al., 1999), depends on post-translational hydroxylation of HIF1α at proline residues 564 and 402 by prolyl hydroxylases (Epstein et al., 2001; Ivan et al., 2001). Oxygen is required for the activity carried out by prolyl hydroxylases.

When hypoxic conditions arise, prolyl hydroxylation ceases, HIFα is not recognized by the pVHL and therefore evades degradation (Berchner-Pfannschmidt et al., 2004). Consequently HIFα dimerizes with HIFβ , and is then translocated to the nucleus where it is bound to the HIF responsive elements (Myllyharju & Schipani, 2010). Formed active complexes turn on expression of target genes including vascular endothelial growth factor (VEGF), erythropoietin, glucose transporter-1, and carbonic anhydrase (Bracken, Whitelaw, & Peet, 2003; Wenger, 2002).

Beak et al. (2005) demonstrated the role of OS9 as a regulator of HIF1. OS9 interacts with HIF1α to promote oxygen-dependent degradation of HIF-1α by the proteasome. Beak's research showed OS9 as a negative regulator of HIF1 that promotes prolyl hydroxylation by interacting with HIF-1α and PHDs. A complex is formed with HIF1α, OS9, and PHD2 or PHD3. OS9 PHD2 binds separately to HIF-1α at non-overlapping sites. OS9 promotes the interaction of HIF-1α with PDH leading to hydroxylation followed by proteasomal degradation. While OS9 has been shown to interact with OS9, it's not known if OS9 is a meprin substrate and how meprin cleavage of OS9 would impact the hypoxia response.

CHAPTER 3

Materials and Methods

3.1 Reagent Inventory

The following antibodies were purchased from Abcam, Cambridge, MA: anti-GAPDH mouse monoclonal antibody, and anti-HIF1α rabbit polyclonal antibody. Minimum essential medium eagle were purchased from the American type culture collection ATCC (Manassas, VA). The following supplies were purchased from Bio-Rad Laboratories, Hercules, CA: 30% acrylamide (29:1 bis solution), anti-mouse IgG secondary antibody, anti-rabbit IgG secondary antibody, and Bio-Rad protein assay reagent. A biotinylated protein ladder was purchased from Cell Signaling Technology (Danvers, MA). Dr. Judith Bond of the Pennsylvania State University College of Medicine (Hershey, PA) donated anti-meprin-A rabbit polyclonal antibody (HMC14) and anti-meprin-B mouse polyclonal antibody (HMC77). The following chemicals were purchased from Fisher Scientific (Pittsburgh, PA): ammonium persulfate, dithiothreitol, ethylenediaminetetraacetic acid, protein markers , glycerol, glycine, hydrochloric acid, hydroxyethyl piperazineethanesulfonic acid, isobutyl alcohol, magnesium chloride, methanol, nonfat powdered milk, phosphate buffered saline, potassium chloride, sodium vanadate, Tris, Tris base, trypsin, tween 20, and β-mercaptoethanol. Antibiotic-antimycotic and fetal bovine serum were purchased from Hyclone Laboratories (Logan, UT). Dulbecco's minimum essential medium was purchased from Life Technologies (Grand Island, NY). Anti-OS9 rabbit polyclonal antibody and purified human OS9 protein were purchased from OriGene Technologies (Rockville, MD). The following chemicals were purchased from Sigma-Aldrich, St. Louis, MO: actinonin, antimycin A, bromophenol blue, cobalt II chloride hexahydrate, sodium bicarbonate, sodium chloride, sodium dodecyl sulfate, tetramethylethylenediamine, tris (2-carboxyethyl)

phosphine hydrochloride, triton X-100, and 2-deoxy-D-glucose. The following chemicals were purchased from Thermo Scientific, Waltham, MA: ethyl alcohol 200 proof (Acros Organics), 100X ethylenediaminetetraacetic acid solution, 100X halt protease inhibitor cocktail, and Supersignal West Pico Chemiluminescent substrate.

3.2 Cell Culture

Human Embryonic Kidney 293 (HEK 293) cells were purchased from American Type Culture Collection (Manassas, Virginia) with the addition of meprin transfected cells gifted from the laboratory of Dr Judith Bond (Penn State College of Medicine, Hershey, PA). Cells were propagated in minimum essential medium eagle supplemented with 10% FBS and antibiotics, subcultured, and preserved in liquid nitrogen. The cell cultures were incubated at 37°C and 5% CO_2 for the duration of the growth cycle. The cell cultures were observed on the second day using an inverted microscope. The cell cultures were observed daily to monitor the confluency of the cells. The cell cultures were replaced with new complete growth media weekly. The cells were grown to a confluency rate of at least 80% before being harvested.

3.3 Oxygen Depletion

Oxygen depletion was induced in the HEK 293 cells using Cobalt (II) Chloride. Cells were allowed to grow to 80% confluency in Eagle's MEM medium with 10% FBS. The media was changed to a serum-free medium containing 0.1% BSA overnight. The cell cultures were exposed to 125 µM cobalt chloride (Sigma, St. Louis, MO) for pre-determined time intervals of 0 min, 30 min, 1 hr, 2 hr, and 3 hr.

3.4 ATP Depletion

A combination of 2-deoxy-D-glucose and antimycin A was used to induce ATP depletion in the HEK 293 cells. The cells were washed 2-3 times with warm, sterile-filtered PBS media. The cell culture medium was replaced with Dulbecco's PBS "ATP-depletion medium" (Life Technologies, Grand Island, NY) containing 1.5 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM deoxy-D-glucose, and 10 µM antimycin A for 0 hr, 30 min, 1 hr, and 2 hr.

3.5 Protein Extraction

A hypotonic buffer, a high salt buffer, and RIPA buffer were used for fractionation of proteins from the cultured cells. The hypotonic buffer consisted of 10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1 mM Na_3VO_4, and protease inhibitor/EDTA. The high salt buffer was prepared by combining the following: 20 mM Hepes (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 1 mM Na_3VO_4, 25% glycerol, and protease inhibitor/EDTA. The RIPA buffer was prepared by combining 0.02 mM Hepes pH7.9, 0.015 mM NaCl, 0.1 mM Triton-X 100, 0.01 mM SDS, 1 mM Na_3VO_4, and protease inhibitor cocktail/EDTA. Cells were rinsed with ice cold PBS twice. The last wash was aspirated completely. Three-hundred µl of hypotonic buffer was added to each 100 mm dish. The dishes were allowed to sit on ice for 30 minutes, and swirled every 10 minutes. The cells were harvested using a cell scrapper. The lysate was transferred to a microfuge tube, and centrifuged at 4° C for 10 minutes at a speed of 15,000 x g. The supernatant was transferred to a new tube, and labeled as the cytoplasmic extract. Fifty µl of high salt buffer was added to the pellet, and the pellet was resuspended in the solution. This was placed on ice for 20 minutes, and centrifuged once again 4° C for 10 minutes at a speed of 15,000 x g. The supernatant was transferred to a new tube and labeled as the nuclear-enriched extract. The pellet was resuspended in 100 µl of RIPA buffer, and allowed to

sit on ice for 20 minutes. The microfuge tube was centrifuged 4° C for 10 minutes at a speed of 15,000 x g. The supernatant was transferred to a new tube labeled as membrane-enriched fraction. All fractions were kept on ice if protein quantification was to be immediately performed, or frozen at -20° C until a later date when the protein quantification was to be performed.

3.6 Determination of Protein Concentration

The concentrations of proteins obtained from the protein extraction were determined using Bio-Rad's protein assay reagent (Hercules, CA). This assay is based upon the Bradford method. The prepared samples were allowed to sit at room temperature for 10 minutes before having their optical densities read on VIS light at 595. Microsoft spreadsheet was utilized in order to plot the standard curve. The estimated protein concentrations were derived from the standard curve.

3.7 Electrophoretic Separation of Proteins by SDS-PAGE

Proteins were separated by electrophoresis on 10% polyacrylamide gels. Equal amounts of proteins ranging from 40-60 µg were loaded in each well. Five µl EZ-Run Pre-Stained Rec Protein Ladder™ (Fisher Scientific, Pittsburgh, PA) was loaded into the first well and used for tracking protein migration. The amounts required of each were obtained from using a pre-formed excel template. The samples were boiled in SDS sample buffer with β mercaptoethanol for 5 minutes before loading. The gels were run at 200 V for roughly 45 min or until the migration front had reached the bottom of the gel.

3.8 Western Blot Analysis

The polyacrylamide gel containing the separated proteins was positioned on nitrocellulose membrane that was presoaked in transfer buffer (250 nM Tris, 192 nM Glycine, and 20% Methanol), and embedded between two thick, pieces of soaked filter paper. The unit was fully assembled, and allowed to transfer at 15 V for one hour. The membrane was incubated in 8% milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T) for one hour at room temperature to block non-specific binding sites. The membrane was incubated in primary antibody with gentle agitation at room temperature for one hour, or overnight at 4° C. The membrane was then washed with TBS-T three times for 10 minutes per cycle at room temperature on a shaker. The secondary antibody was added using the same procedure with the exception that the secondary antibody is added in TBS-T in lieu of milk. The membrane underwent its final wash with TBS-T with three cycles at 15 minutes each.

The Western blots were developed using Chemiluminescent Substrates (Thermo Scientific, Waltham, MA). The film was processed using the X-ray developer. The quantitation of the protein bands was performed using Bio-Rad's GS-800 calibrated densitometer along with QuantityOne software.

3.9 Confirmation of Meprin B Expression by Meprin β cDNA Transfected Cells

In order to assess the interaction of meprin with OS9, and HIF1α, it was necessary to verify the presence of meprins within the transfected cells. Using Western blot analysis, meprin B was detected in the cytosolic and membrane-enriched fraction (Figure 1). Furthermore, the detection of meprin within the cytosolic fraction provides evidence that OS9 and meprin can be found within close proximity to one another within the cell.

3.10 Determination of OS9 Cellular Localization

Western blot analysis were performed on all three cell genotypes HEK 293 cells to determine the cellular localization for OS9. OS9 was strongly detected in the nuclear fraction for all of the cell proteins. Faint bands were also detected within the cytosolic fraction. This information provided the basis for only using the nuclear fraction for carrying out the Western immunoblots when investigating OS9.

3.11 Determining If Meprins Degrade OS9

To determine if meprins are capable of cleaving OS9, purified activated forms of meprin A and meprin B (4 nM) were co-incubated with purified OS9 (92 nM) in buffer consisting of 20 mM Tris and 150 mM NaCl, pH 7.5. Control reactions were incubated in buffer without meprins. Equal volumes of proteins were taken from the reaction mixture at 0, 0.5, 1, 2, 3, and 4 hours. The reaction was stopped by addition of SDS buffer and boiling for 5 minutes. The samples were loaded onto 10% polyacrylamide gels and separated by electrophoresis as described above, followed by Western blot analysis with anti-OS9 antibodies.

3.12 Determining Whether Degradation of OS9 Is Meprin Isoform-Specific

To confirm that degradation of OS9 was meprin-specific, purified OS9 was co-incubated with activated meprin B as described above. Controls reactions were included with the following; (1) activated meprins that were pre-incubated with known meprin inhibitors (EDTA and actinonin) for 1 hour, (2) latent forms of meprin B, and (3) trypsin-treated buffer. The reactions were incubated at 37^{o}C for 1 hour and the proteins separated by electrophoresis and detected by Western blot analysis.

CHAPTER 4

Results

4.1 Cellular Localization of Meprin B and OS9

Western blot analysis showed that meprin B proteins were present in the membrane-enriched and cytosolic-enriched protein fractions from the meprin β transfected cells (Figure 1). This data confirmed that the cells are expressing meprin. The confirmation of meprin within the cytosolic fraction places meprin in close proximity to OS9.

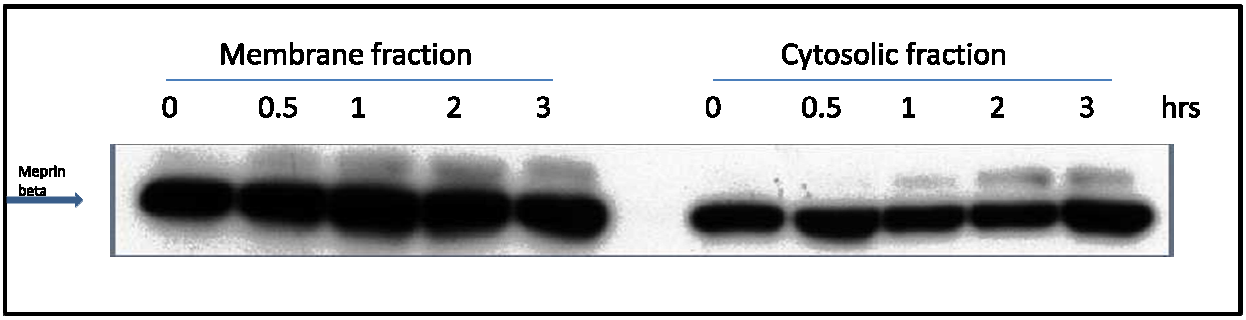

Figure 1. Evaluation of meprin B expression in meprin β transfected MDCK cells.

OS9 proteins were most abundant in the nuclear-enriched protein fraction from all three cell genotypes (non-transfected, meprin alpha-transfected, and meprin beta-transfected) HEK293 cells. Western blot analysis revealed traced amounts of OS9 within the cytosolic fraction of the non-transfected cells as seen in Figure 2. Determining that the nuclear fraction contained the most amount of OS9 from all cell genotypes provided the basis for only using the nuclear fraction for the protein samples used in all subsequent Western blots.

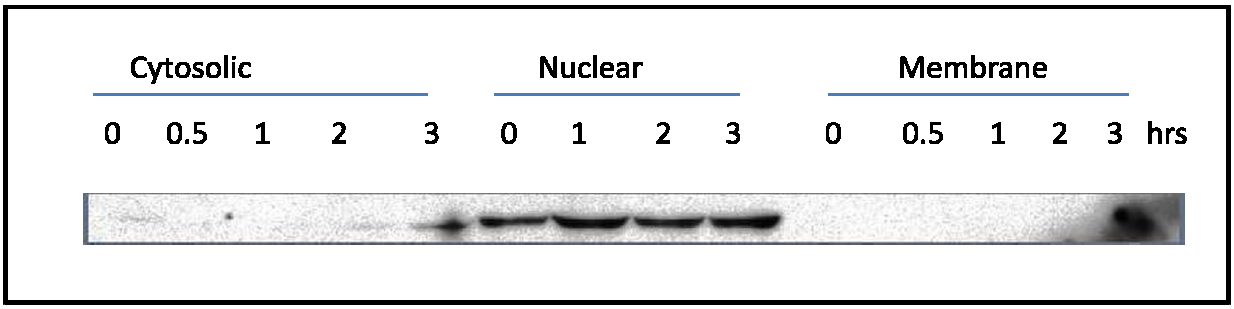

Figure 2. Cellular localization of OS9.

4.2 Increase of OS9 in Oxygen-Depleted Non-Transfected HEK293 Cells

With the location of OS9 being established within the cell fraction and the cells evaluation for the expression of meprin, all genotypes of HEK 293 cells were treated for oxygen-depletion with the purpose of evaluating the presence of OS9 within the nuclear fraction. OS9 was observed at all time intervals for all of the HEK 293 genotypes. Evaluating the data from the non-transfected cells only, OS9 increased over the course of the treatment. Both the alpha transfected and beta transfected HEK 293 cells did not change over time (Figure 3).

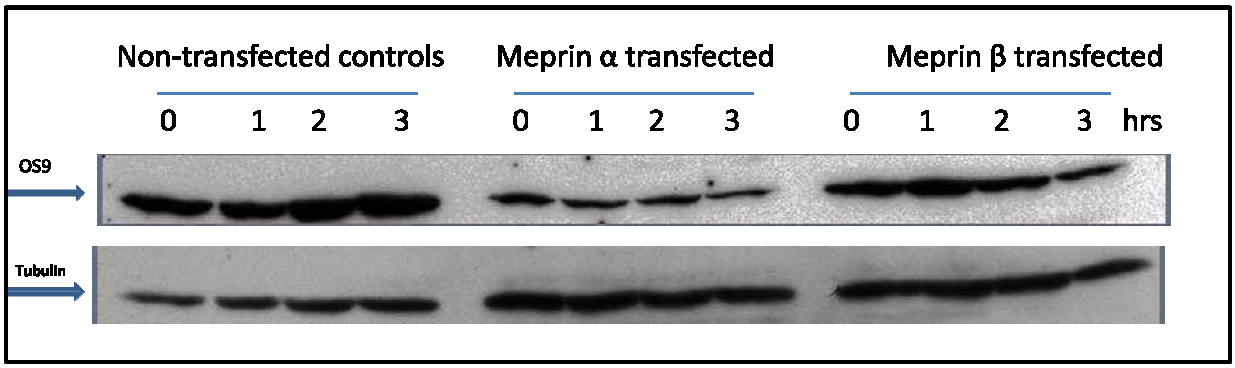

Figure 3. Increase of OS9 in oxygen-depleted non-transfected HEK293 cells.

4.3 Time-Dependent Stabilization of HIF1α in Oxygen-Depleted HEK293 Cells

In addition to performing Western blots of OS9 in oxygen-depleted cells, nuclear levels of the hypoxia response gene HIF-1α were also evaluated. As shown in Figure 4, no HIF-1α was

observed at the zero hour time point with HIF-1α having trace amounts after 1 hour of oxygen-depletion. A significant amount of HIF-1α was detected after 2 and 3 hours of oxygen-depletion for all of the nuclear extracts. It was determined that HIF-1α stabilized in a time-dependent manner. When comparing the amount of HIF-1α detected in the non-transfected control cells to the meprin β transfected cells, much more HIF-1α was detected in the transfected cells with the most HIF-1α occurring in the beta-transfected cells (Figure 4).

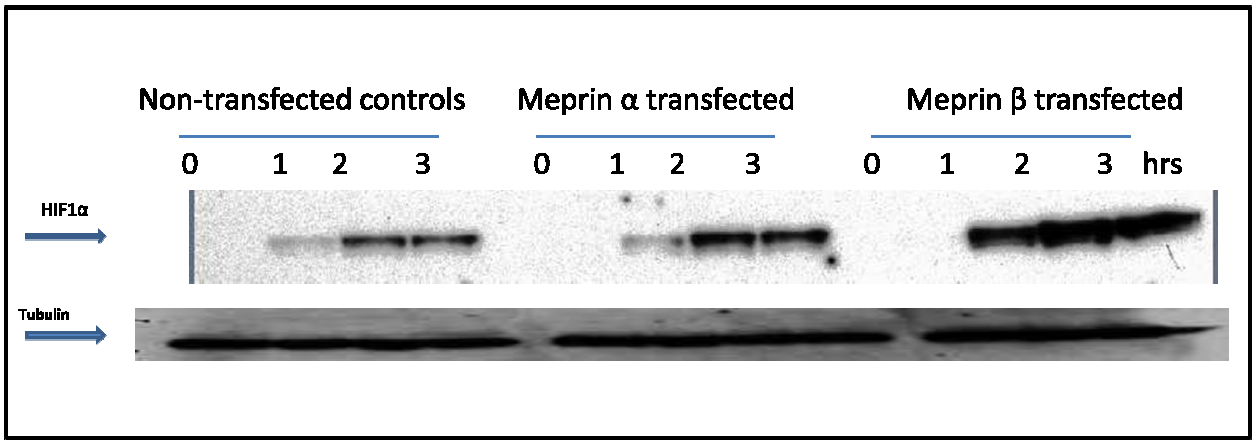

Figure 4. Time-dependent stabilization of HIF-1α in oxygen-depleted HEK293 cells.

These results were obtained from two independent experiments different times. Faint bands for HIF-1α were observed at zero hours on one occurrence. It was expected that HIF-1α may or may not be detected at the time point of 0 hour due to degradation of HIF-1α in a normoxic state.

4.4 Interaction Between OS9 and HIF1α in Hypoxia

Combining the data obtained from the Western blots performed on nuclear fractions of HEK293 cells depleted of oxygen, a correlation between OS9 and HIF1α was suggested. A time-dependent stabilization of HIF1α occurred within the nuclear fractions for all 3 genotypes.

An increase in OS9 was only observed in the non-transfected cells. A relationship appears to exist between OS9 and HIF1α as a response to hypoxia.

4.5 Evaluation of HIF1α in ATP-Depleted MDCK Verses MDCK Beta Transfected Cells

Non-transfected MDCK and MDCK cell transfected with meprin β cDNA were depleted of ATP using pharmacological agents for 0, 0.5, 1, 2, and 3 hours. It was observed in both genotypes that HIF-1α stabilized over time when deprived of ATP (Figure 5).

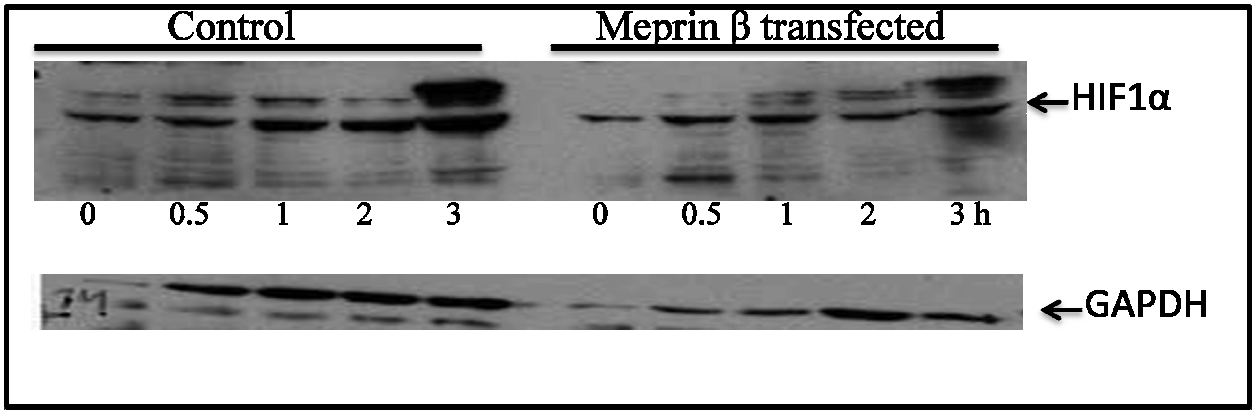

Figure 5. Evaluation of HIF-1α in ATP-depleted MDCK verses MDCK beta transfected cells.

4.6 Meprin B Degradation of OS9

In order to determine if meprin cleaves OS9, homomeric active forms of meprin A and B were incubated with purified recombinant human OS9 for O hr, 0.25 hr, 0.5 hr, 1 hr, 2 hr, and 4 hours. A buffer containing no meprin served as a control. The samples were subjected to electrophoresis, and a Western blot was performed to detect OS9 using anti-OS9 antibodies. Intense bands of OS9 were observed for the samples prepared with the control buffer (no meprin) and for the samples containing meprin A at all time points. For the reactions containing meprin B, the intensity of the OS9 band greatly decreased after 30 minutes of co-incubation resulting in an extremely faint OS9 band at 1 hr. No OS9 was detected at the time points of 2 and 4 hours. This demonstrates that OS9 is being degraded by meprin B. Figure 6 displays the

results for no meprin, meprin A, and meprin B incubation with purified OS9. OS9 was detected amongst all the samples containing no meprin (Figure 6). The samples incubated with meprin B revealed one intense band for OS9 at O hour, and no bands were observed for any of the other time points. This data suggests that meprin B degrades OS9.

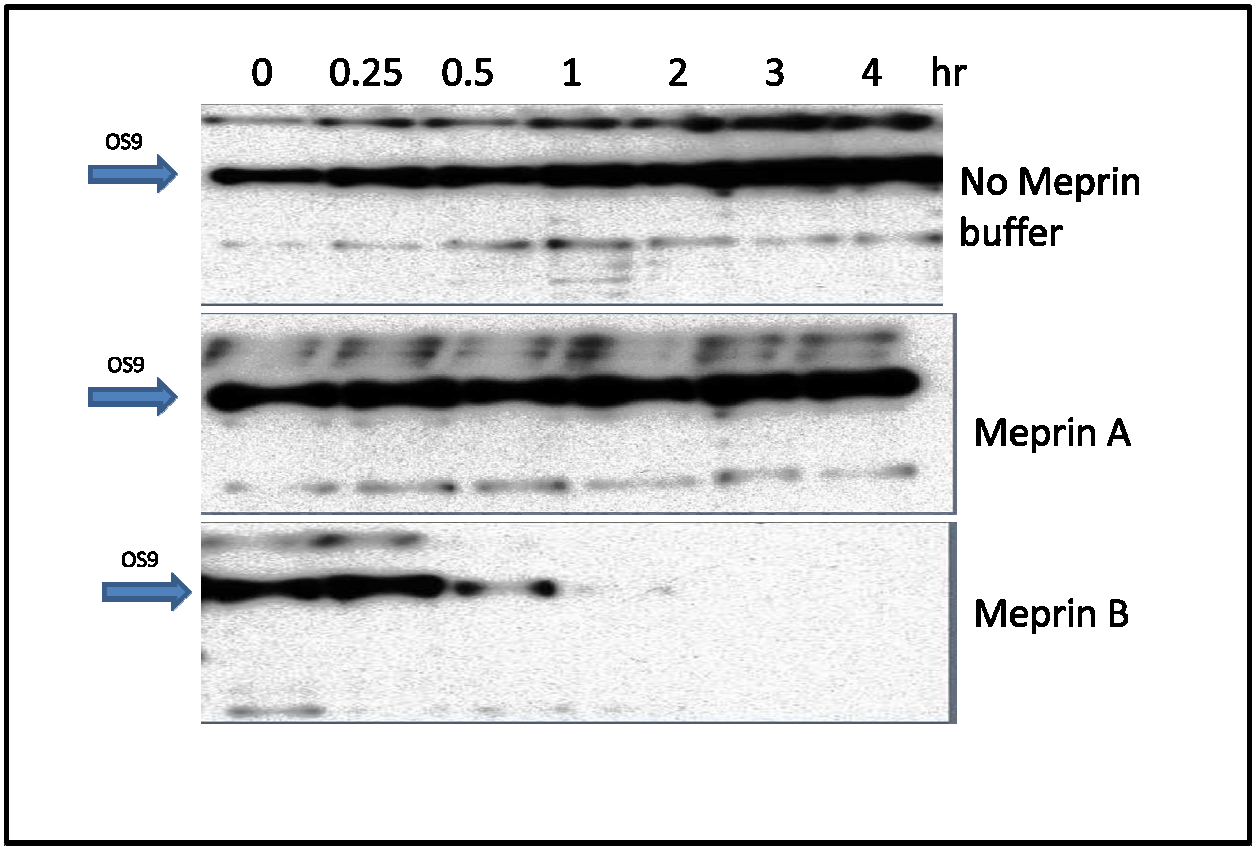

Figure 6. Meprin B cleaves/degrades purified OS9.

4.7 Meprin B Degradation of OS9 Is Meprin Specific.

The specificity of meprin cleaving OS9 was evaluated by incubating purified recombinant human OS9 with activated meprins, latent forms of meprins, buffer containing no meprin, and buffers containing meprin inhibitors. The samples were run out on a gel, and a Western blot for OS9 was performed. Intense bands for OS9 were detected in the samples containing OS9 with meprin-free buffer, OS9 with the latent form of meprin, and OS9 with the

inhibitor of EDTA. Extremely faint bands were detected in the samples containing OS9 with the activated form of meprin A, and OS9 with the inhibitor actinonin. The data supports that the activated forms of meprin degrades OS9 resulting in the observation of no bands, and demonstrating that meprin B degradation of OS9 is meprin specific (Figure 7).

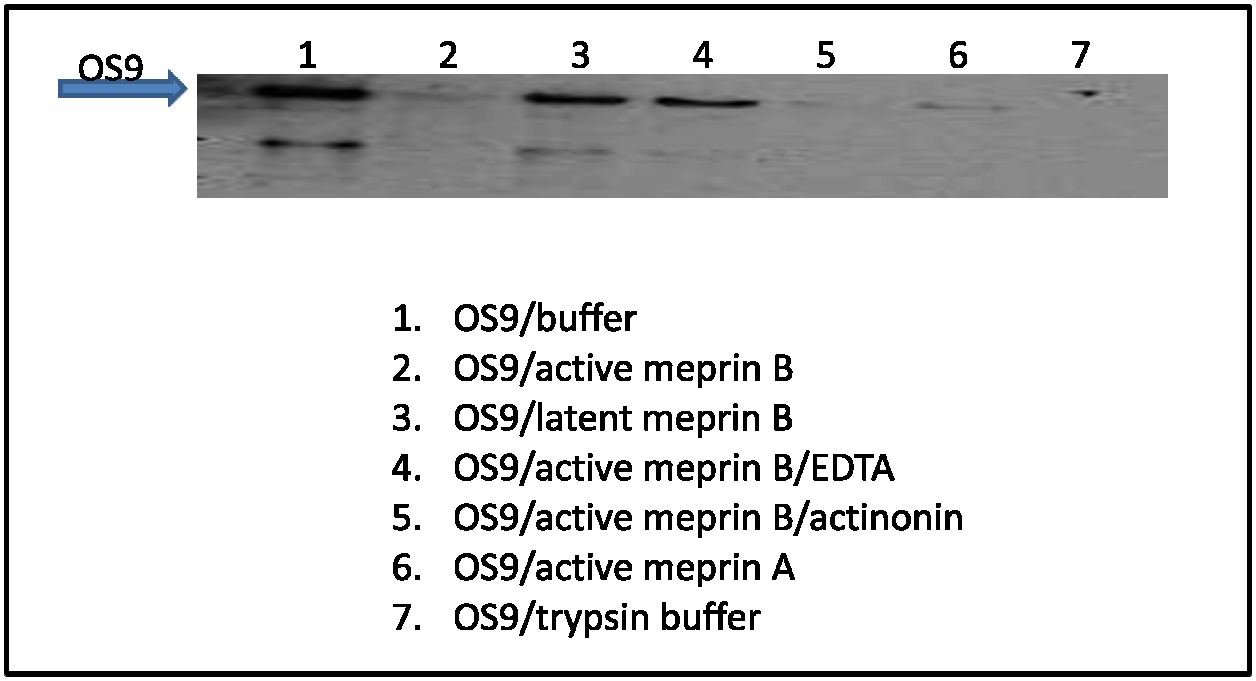

Figure 7. Meprin B degradation of OS9 is meprin specific.

CHAPTER 5

Discussion and Conclusion

Ischemia-reperfusion injury is the leading cause of acute kidney disease (Gunaratnam & Bonventre, 2009). On average, 2.5% of hospitalized patients will die from complications resulting from ischemic acute tubular necrosis (Ympa et al., 2005). It is necessary to determine what is causing the damage inside the organ when normal blood flow is restored after a period of ischemia and hypoxia. Meprins constitute 5% of the total amount of proteins found in the kidneys' brush-border membrane, and meprins have been associated in the pathology of ischemia-reperfusion induced kidney failure. It has been shown in mice that lower levels of meprin and no meprin (knockout) lead to less renal injury after ischemia/reperfusion. This research provided another link in the mechanism for how meprins enhance damage to ischemia-reperfusion injured kidney.

An interaction between OS9 and meprin B has previously been described by Litovchick et al. (2002) suggesting that OS9 may be involved in ER to Golgi transport of meprin B. A gap was created when OS9 has never been identified as a meprin substrate. The goal of this study was to determine if OS9 is a meprin substrate. The identity of a meprin substrate will provide to a better understanding of how meprins are enhancing damage to the kidneys after an episode of ischemia-reperfusion injury; thus, yielding to developments in the prevention and treatment of kidney damage resulting from ischemia-reperfusion injury.

To test if OS9 is a meprin substrate, purified human OS9 was co-incubated with meprin A, meprin B, or no meprin. Results revealed that only meprin B degrades OS9. When the experiment was repeated using less sample, no OS9 was observed at any time points after 0 hour.

This suggests that not only is OS9 being degraded by meprin B, but it is also fast acting. An enzyme has more potential to create damage when the rate of reaction is rapid.

The degradation of OS9 by meprin was further investigated using various experimental variables to determine the specificity of meprin degradation of OS9. OS9 was incubated with activated and latent isoforms of meprin, and with known meprin inhibitors. It was determined that the degradation of OS9 is specific to activated meprin B. This research is the first to identify OS9 as a meprin substrate, and that degradation only occurs by meprin B.

Meprins relocalize from the brush-border membrane to the cytosol after ischemia-reperfusion. It was confirmed by Western blot that meprin is found within the cytosolic fraction. Meprin being found within the cytosol puts the meprin in close proximity to OS9 located on the cytoplasmic side of the ER. If meprin was not found within the cytosol, the potential would not exist for OS9 and meprin to interact with one another.

OS9 is associated with the ER-associated degradation (ERAD) pathway machinery and ERAD substrates. Misfolded proteins are recognized by the ERAD pathway, and retranslocated back across the ER membrane in order to be degraded by the cytosolic proteasome. OS9 binds to the misfolded polypeptide within the lumen of the ER before transferring the protein to the HRD1 ubiquitin ligase complex transfer and polyubiquitination. OS9 degradation by meprin B would inhibit the ERAD pathway from occurring. With the ERAD unable to eliminate misfolded proteins, the proteins would begin to build up within the cell. The accumulation of protein within the fragile cell could lead to additional damage. Higher levels of peptides are observed in patients with chronic renal failure when their kidneys are unable to provide clearance. Data from the current study suggests that the mechanism of damage

resulting to the kidney upon ischemia-reperfusion is in part the result of the trafficking of proteins being halted when meprin B degrades OS9.

The second objective of this study was to determine whether OS9 interacts with meprins and HIF1α under hypoxic conditions. To date, there is no known correlation existing between OS9 and HIF1α. Hypoxia occurs as a result of ischemia. A cell responds to the low oxygen level via inducing hypoxia inducible factors with HIF1 functioning as the master regulator of oxygen homeostasis. Beak et al. (2005) demonstrated that OS9 interacts with HIF1α and HIF1α prolyl hydroxylases acting as a negative regulator of HIF1 in oxygen homeostasis. Using RNAi, Beak et al. discovered OS9's gain-of-function to include HIF1α hydroxylation, HIF1α proteasomal degradation, and HIF1-mediated degradation. OS9 loss-of-function included increase levels of HIF1α, and HIF1-mediated transcription. Taking this into consideration, an increased amount of HIF1α would be expected in cells depleted of oxygen and exposed to meprins. The results obtained from this study found that HIF1α stabilizes in a time-dependent course when cells are depleted of oxygen. This is the first time that HIF1α is evaluated in cells transfected with meprin. An increase in HIF1α was observed in the alpha transfected cells compared to the non-transfected cells. The biggest increase in the amount of HIF1α was detected among the beta transfected cells. This would be expected if OS9 is being degraded by meprin B. The degraded OS9 is no longer able to interact with HIF1α and HIF1α prolyl hydroxylases to promote degradation of HIF1α. The extra accumulation of HIF1α protein could potentially result in additional damage to the cell.

In conclusion, this research established that OS9 is a meprin B substrate. Meprin A did not cleave or degrade OS9 suggesting isoform-specific substrate preference. HIF1α was shown to be affected by the degradation of OS9 by meprin B; thus, impacting the hypoxia response of

the cell. It was proposed that cell damage observed in ischemia-reperfusion injury may be partially the result of the ERAD pathway being modulated when OS9 is degraded by meprin B.

CHAPTER 6

Future Research

The next step to be performed to continue working on this project is concluding the ATP-depletion experiments in the HEK293 cells. Preliminary data using MDCK cells were obtained, but due to time restraints, ATP-depletion portion of this project is not complete. The protocol had to be modified due to cells being lost upon extraction. The new protocol for extracting cells has been proven to be successful, and is ready to be carried out. Three Western blots each for OS9 and HIF1α will provide statistically sound data that will be ready to publish. Also, the data obtained from *in vitro* results can be combined and compared to *in vivo* results from the mice studies.

Immunohistochemistry for OS9 and HIF1α in the HEK293 cells depleted of oxygen and ATP would be the last component of this project. The visualization would provide direct evidence to what is occurring to OS9 and HIF1α within the cell. Additionally, a commercially available ERAD pathway inhibitor could be purchased to compare non-transfected cells to meprin β transfected cells. The meprin B degradation resulting in the stop of protein trafficking in the ERAD pathway should result in the same findings where compared to an ERAD pathway inhibitor in the non-transfected cells. Immunohistochemistry would serve purposely for ERAD pathway as well.

In the near future, a large scale search for additional meprin substrates can be performed using techniques such as microarrays and mass spectrometry. The ultimate goal is to not treat kidney disease, but to prevent damage and disease. The identification of meprin substrates will aide in drug development. Meprin inhibitors such as actinonin are now available, but they are

not meprin specific. The identification of all meprin substrates would lead to better drugs that could be used in personalized medicine.

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