2018

Role of N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) and renal hemodynamics on obesity related renal damage

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ROLE OF N-ACETYL-SERYL-ASPARTYL-LYSYL-PROLINE (Ac-SDKP) AND RENAL HEMODYNAMICS ON OBESITY RELATED RENAL DAMAGE

A dissertation submitted to
the Graduate College of
Marshall University
In partial fulfillment of
the requirements for the degree of
Doctor of Philosophy
In
Biomedical Sciences
by
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Marshall University
August 2018

APPROVAL OF DISSERTATION

We, the faculty supervising the work of Mani Maheshwari, affirm that the dissertation, *"Role of N-Acetyl-Seryl-Aspartyl-Lysyl-Proline (Ac-SDKP) and Renal Hemodynamics on Obesity Related Renal Damage"* meets the high academic standards for original scholarship and creative work established by the Biomedical Science Program and the Graduate College of Marshall University. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

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DEDICATION

I would like to dedicate my work to my parents, Mukesh Maheshwari and Madhuri Maheshwari for their unconditional love, motivation, and encouragement. Their untiring support and unfailing trust in me are the reason of what I become today. To my husband, Dr. Sumit Ranjan Monu for his love, patience and understanding my challenges of graduate life. His continuous support, help and motivation have given me immense strength to remain strong despite many obstacles and make this dream a reality. Finally, to my Son, Neil Verma for all his love and support he has shown to make it happen.

ACKNOWLEDGMENTS

I would like to thank my mentor Dr. Oscar A. Carretero for his guidance, encouragement and support. I am very much fortunate and privileged to receive guidance and help from him. I express my wholehearted indebtedness to him for his interesting ideas and discussions that were profoundly fruitful. His scientific acumen, critical judgments and trust in my abilities has guided and motivated me throughout the course of this investigation and compilation of manuscript. I consider myself fortunate to have worked under him. I sincerely thank him for his transcendent suggestions and efforts to embellish this research.

I would like to thank my Committee Chair Dr. Richard Egleton for accepting to be a chair on my committee and being very supportive, and other members of my dissertation committee, Dr. Todd Green, Dr. Elsa I. Mangiarua, and Dr. Travis Salisbury for their constructive feedback. I am deeply grateful to them for all the support they have given me. Their valuable suggestions at many critical junctures of my work helped me reach this step.

Special thanks to Dr. Green for all his cooperation and support. He has always been very prompt in giving all the replies of my queries. My transition to Henry Ford from Marshall University followed by the time I took to complete my dissertation wouldn't have been so smooth without his support.

I owe my sincere thanks to Dr. Cesar Romero and Dr. Nitin Kumar for teaching me numerous lab techniques. Those skills have been invaluable in this project. Without your skillful assistance this thesis would not have completed. I value your scientific enthusiasm and positive attitude.

I wish to thank sincerely all of my co-authors and collaborators for making this work possible. Dr. Sumit Ranjan Monu, for helping me with my experiments and for scientific inputs, Dr.Tang-Dong Liao for expertise and help with immunohistochemical analyses.

I would also like to thank the Hypertension and Vascular Research Division at Henry Ford Hospital to allow me to conduct my research at the hospital. I am very thankful to Carl Polomski for all his help throughout the time I spent in the lab. I would also like to thank Gulser Gurocak for help with enzyme immuno assays and also for her motherly perspective

I would like to place on record my gratitude towards Marshall University for giving me the opportunity to earn a doctoral degree.

I feel short of space to express my sincere gratitude to my friend Shashwati Mathurkar who has always been there for me during this journey of my PhD. Her affection, moral support and constant encouragement have helped me immensely to carry out my work.

Last but not the least; my warmest thanks go to my siblings, Priyanka & Manish. Their affection, support and constant encouragement have always been my strength, which has helped in all these years to reach where I am today.

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ABSTRACT

Obesity is a public health problem and is associated with salt-sensitive hypertension, kidney inflammation and fibrosis. N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) is a tetra-peptide with anti-inflammatory and anti-fibrotic properties but its effect on kidney damage in obesity is unknown. We hypothesized that high salt fed Zucker obese (ZO) rats develop renal damage, inflammation and fibrosis and that Ac-SDKP prevents these changes. Zucker lean (ZL) rats served as controls. Animals were treated with Ac-SDKP while maintained on either a normal-salt or HS diet for 8 weeks. Systolic blood pressure (SBP), albuminuria, renal inflammation and fibrosis were evaluated. HS diet increased macrophage infiltration in the kidneys of both ZL and ZO rats but was significantly higher in HS fed ZO rats. Ac-SDKP prevented macrophage infiltration in ZO rats. Similarly, glomerulosclerosis, cortical and medullary interstitial fibrosis were increased in ZO rats fed the HS diet, and Ac-SDKP attenuated these alterations. SBP was increased in HS-fed ZO rats, and was significantly decreased with Ac-SDKP treatment. Ac-SDKP treatment failed to improve albuminuria ZO rats. Conclusion: Ac-SDKP treatment in HS-fed ZO rats prevented renal damage by reducing inflammation, fibrosis, and SBP. Additionally, we studied the renal hemodynamics in ZO rats. ZO rats have higher glomerular capillary pressure (P_{GC}) that can cause renal damage. P_{GC} is controlled by the afferent arteriole (Af-Art) resistance which in turn is regulated by two intrinsic feedback mechanisms, tubuloglomerular feedback (TGF) that causes Af-Art constriction and connecting tubule glomerular feedback (CTGF) that causes Af-Art dilatation in response to an increase in sodium chloride (NaCl) transport in the connecting tubule *via* the epithelial sodium channel (ENaC). Since CTGF is a dilatory mechanism, we hypothesized that increased CTGF contributes to TGF attenuation and decreases P_{GC} in ZO rats. We measured stop-flow pressure (P_{SF}), surrogate of P_{GC} in ZO rats

using in-vivo renal micropuncture. Maximal TGF response was attenuated while CTGF was elevated in ZO rats compared to ZL rats. CTGF inhibition with ENaC normalized the maximum P_{SF} change in ZO rats indicating an important role of CTGF in TGF attenuation. Conclusion: enhanced CTGF contributes to TGF attenuation in ZO rats and potentially contributes to progressive renal damage.

CHAPTER 1

INTRODUCTION

According to the National Institute of Health, obesity should be considered the most important factor for the end-stage renal disease due to its strong association with diabetes and hypertension. Furthermore, incidence of obesity related kidney damage has increased 10-fold in the decade from 2005 to 2015 and is expected to rise further in the coming years (Kovesdy, Furth, & Zoccali, 2017; Mathew, Okada, & Sharma, 2011). In the United States alone, almost 70% of the population is overweight and among them approximately 35% of the people are obese with body mass index above 30 kg/m^2 (J. E. Hall, do Carmo, da Silva, Wang, & Hall, 2015). Obese individuals have been also linked to the salt sensitive hypertension both clinically and in experimental settings (Ali, Patel, & Hussain, 2015; DeMarco, Aroor, & Sowers, 2014). In the obese population, salt-sensitivity is strongly associated with progression of hypertensive target-organ damage, including end-stage renal disease (Quigley et al., 2009). Obesity predisposes the population to be hypertensive and initiates a cascade of associated cardio-renal and metabolic disorders. The Framingham Study by Garrison *et al*. suggests that almost 70% of essential hypertension can be due to excessive weight gain (Garrison, Kannel, Stokes, & Castelli, 1987). Rising incidence of obesity is a crucial factor for the increased incidence of diabetes and hypertension that is a major risk factor for cardiovascular and renal disease. The underlying mechanism of obesity related salt sensitivity and its association with renal injury remains unclear. However, inflammation is thought to play a key role in the development of obesity related kidney damage (Harrison et al., 2011; Schiffrin, 2014). Previous studies have shown that obesity induced renal injury is associated with increased albuminuria, infiltrating immune cells,

tubulointerstitial injuries, and glomerulosclerosis and it gets further aggravated by high salt diet (Dobrian, Schriver, Lynch, & Prewitt, 2003; M. E. Hall et al., 2014).

N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) is a naturally occurring tetra-peptide that is released from its precursor thymosin β4, by two enzymatic steps mediated by meprin-α and prolyl oligopeptidase (Cavasin, Rhaleb, Yang, & Carretero, 2004; Kumar et al., 2016). Ac-SDKP is found in human plasma, circulating mononuclear cells (Pradelles et al., 1990) and various other organs in the body (Junot et al., 1999). Ac-SDKP is hydrolyzed mainly by angiotensin converting enzyme (ACE) and its endogenous levels are increased by ACE inhibitors (ACEi) in the plasma, urine, kidney, and heart (Azizi et al., 1996). We previously demonstrated that part of the anti-inflammatory and anti-fibrotic effects of ACEi are mediated by an increase in endogenous Ac-SDKP (Peng, Carretero, Liao, Peterson, & Rhaleb, 2007; Peng et al., 2005). Studies in several experimental animal models have demonstrated that Ac-SDKP has anti-inflammatory and anti-fibrotic properties (Rhaleb, Pokharel, Sharma, & Carretero, 2011; Worou et al., 2015), and that a decrease in endogenous Ac-SDKP levels promoted fibrosis of heart and kidney (Cavasin, Liao, Yang, Yang, & Carretero, 2007). Recently, we have also shown that Ac-SDKP can delay the onset of hypertension in systemic lupus erythematosus (Nakagawa et al., 2017). However, the effect of Ac-SDKP in obesity related kidney damage and hypertension is still unknown.

Clinical trials demonstrated that treatment with ACEis improves clinical outcome in patients suffering from obesity related progressive renal disease (Mallamaci et al., 2011). ACEis ameliorate glomerular hypertension by reducing the efferent arteriole resistance (Bosma, Krikken, Homan van der Heide, de Jong, & Navis, 2006). However, the beneficial effect of ACEi might not only be dependent upon the suppression of renin angiotensin system but also on

other biochemical effects including a peptide known as N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) (Peng et al., 2007; Peng et al., 2005).

We used Zucker obese (ZO) rats to study obesity related kidney damage. This model exhibits many phenotypic traits common for obesity related kidney damage observed in human population and is associated with a progressive decline of renal function and albuminuria (Kasiske, O'Donnell, & Keane, 1992; Kurtz, Morris, & Pershadsingh, 1989). These rats exhibit hyperinsulinemia but are normoglycemic representing the prediabetic state in the human beings. Zucker lean (ZL) rats were used as the control animals in our experiments.

Based on all the findings mentioned above, we hypothesized that in Zucker obese rats on high salt diet Ac-SDKP prevents renal damage by decreasing renal fibrosis, albuminuria, and glomerulosclerosis as well as delays the onset of hypertension.

Additionally, we also studied the role of renal hemodynamics in obesity related kidney damage. Alterations in renal hemodynamics have been implicated as one of the key factors for the renal damage observed in obese individuals, but the mechanisms of the alterations in renal hemodynamics are unknown (Bondar, Klimontov, & Simakova, 2011; Bosma et al., 2006; Leggio et al., 2017). These hemodynamic changes include increased renal blood flow, glomerular capillary pressure (P_{GC}), and glomerular filtration rate (Bondar et al., 2011; Bosma et al., 2006; Sebekova, Klassen, Bahner, & Heidland, 2004). Sustained elevation in P_{GC} in particular can cause stretch in the glomerular cells and cause glomerular barotrauma that can lead to enhanced renal damage (Riser et al., 1992; Sebekova et al., 2004).

In a normal kidney, renal blood flow is tightly controlled due to the existence of renal autoregulatory mechanisms that include tubuloglomerular feedback (TGF), connecting tubule glomerular feedback (CTGF), and myogenic response (Carlstrom, Wilcox, & Arendshorst, 2015;

Monu et al., 2017). Afferent arterioles (Af-Art), glomerular capillaries and efferent arterioles (Ef-Art) are arranged in series, and thus, their dynamics are closely interconnected (Monu et al., 2017).

Arrangement of two resistance vessels, the Af-Art and the Ef-Art, regulate inflow and outflow of blood through the glomerular capillaries, and thus, regulate both P_{GC} and single nephron glomerular filtration rate (Figure 1) (Monu et al., 2017). Af-Art constriction can reduce P_{GC} and glomerular plasma flow downstream that in turn can decrease glomerular filtration. Likewise, constriction of the Ef-Art can build the pressure upstream and may increase P_{GC} and single nephron glomerular filtration rate (Ren, Garvin, & Carretero, 2001; H. Wang et al., 2015). Af-Art resistance is controlled by two renal intrinsic feedback mechanisms: 1) TGF that causes Af-Art constriction in response to increased NaCl in the macula densa, *via* the sodium–potassium-2-chloride cotransporter-2 (NKCC2), and 2) CTGF that causes Af-Art dilatation and is initiated by the epithelial sodium channels (ENaC) in the connecting tubule (CNT) (Figure 1) (Monu et al., 2017; H. Wang et al., 2015).

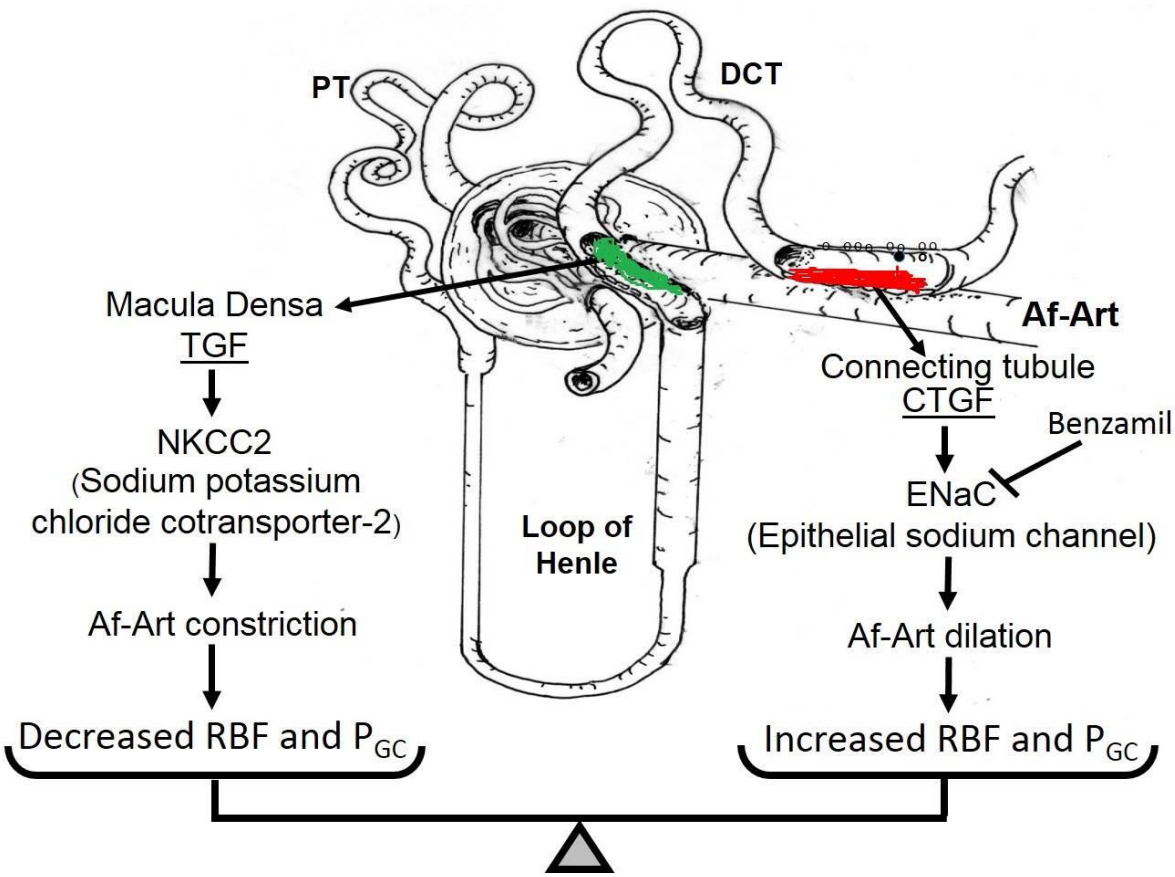

Figure 1. Schematic Representation of the Feedback Mechanisms (TGF and CTGF) in the Kidney. (Af-Art-Afferent arteriole, PT- Proximal tubule, DCT- Distal convoluted tubule, TGF- Tubuloglomerular feedback, CTGF- Connecting tubule glomerular feedback, RBF- Renal blood flow, P_{GC}- Glomerular capillary pressure). © Wang et al. 2013, Hypertension.

Previous studies suggest that there is increased P_{GC} (measured using the stop flow pressure method) in ZO rats (Park & Kang, 1995; Park & Meyer, 1995). TGF attenuation has been hypothesized for the enhanced pressure transmission from systemic circulation to the glomerulus leading to increased P_{GC}, but to our knowledge, no direct study has been done to evaluate the TGF mechanism in obesity. TGF attenuation could make the kidney susceptible to barotrauma and eventual glomerulosclerosis (Azar, Johnson, Hertel, & Tobian, 1977). However, the mechanism of enhanced P_{GC} in obesity is poorly defined.

Since CTGF is a vasodilator mechanism, we therefore investigated whether CTGF plays a role in TGF attenuation in obesity. We used ZO and ZL rats in our study. We hypothesized that

increased CTGF contributes to TGF attenuation, which in turn increases P_{GC} in ZO rats. To test this hypothesis, *in-vivo* renal micropuncture studies were performed in these Zucker rats (8-10 weeks old) using the stop-flow technique.

Aims of the Study

Aim (1). To determine whether Ac-SDKP ameliorates the high salt induced hypertension in obesity.

Aim (2). To determine whether Ac-SDKP ameliorates the high salt induced renal damage by decreasing inflammation and fibrosis in obesity.

Aim (3). To determine if elevated CTGF contributes to TGF attenuation in obesity

CHAPTER 2

REVIEW OF LITERATURE

N-Acetyl-Seryl-Aspartyl-Lysyl-Proline (Ac-SDKP) Synthesis and Metabolism

N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) is a naturally occurring tetra-peptide inside the body, which was originally isolated from the fetal calf bone marrow (Lenfant et al., 1989). Ac-SDKP is found in human plasma, circulating mononuclear cells (Pradelles et al., 1990), and various other organs of the body (Junot et al., 1999). Studies in several experimental models have demonstrated that Ac-SDKP has anti-fibrotic and anti-inflammatory properties (Rhaleb et al., 2011; Worou et al., 2015). The details for the synthesis of endogenous Ac-SDKP are not very clear but most studies suggest that thymosin β4 (Tβ4) is the most likely precursor of Ac-SDKP. Tβ4 contains the Ac-SDKP sequence in its NH2 terminal and is a G-actin-sequestering 43 amino acid long peptide (J. M. Liu et al., 2010). The formation of Ac-SDKP involves enzymatic degradation of Tβ4 in two steps (Figure 2). An enzyme called meprin that releases amino terminal intermediate peptides that are less than 30 amino acids long mediates the first step. Meprin-α is a metalloprotease and is highly expressed in the mammalian kidney and intestine, and it is reported to hydrolyze other peptides and proteins, such as growth factors, peptide hormones and extracellular matrix proteins (Broder & Becker-Pauly, 2013). Meprin-α plays a role in the physiological processes involved in renal and intestinal diseases. Meprin-α has been shown to possess pro-angiogenic properties in both in-vivo and in-vitro studies (Lottaz et al., 2011; Schutte, Hedrich, Stocker, & Becker-Pauly, 2010). Additionally, clinical and animal studies have shown that it has anti-inflammatory properties (Banerjee et al., 2011; Banerjee et al., 2009). The second step is mediated by an enzyme called prolyl oligopeptidase (POP), which acts on these short peptides and leads to the formation of Ac-SDKP (Kumar et al., 2016). POP is

a serine protease present in various organs including kidney, heart, liver, muscles and brain. POP hydrolyzes peptide bonds in angiotensin, substance P, neurotensin, bradykinin, arginine-vasopressin, and oxytocin. POP may play an important role in the biological maturation or degradation of these peptide hormones (Polgar, 2002). POP is involved in a variety of pathophysiological processes such as inflammation, fibrosis and angiogenesis (Cavasin et al., 2004). Ac-SDKP gets hydrolyzed in the presence of Angiotensin Converting Enzyme (ACE). The endogenous levels of Ac-SDKP in plasma are minimal. Ac-SDKP concentration increased fivefold after the administration of ACE inhibitors like captopril and enalapril (Azizi et al., 1996). There are two catalytic domains of ACE, namely C-terminus and N terminus, which cleave the target substrate. Ac-SDKP is hydrolyzed by the N- terminal domain of ACE (Kanasaki, Nagai, Nitta, Kitada, & Koya, 2014).

Additionally, Tβ4 possesses anti-fibrotic and tissue protective effects (Huff, Muller, Otto, Netzker, & Hannappel, 2001). Tβ4 is present ubiquitously in the body and participates in various biological activities (Hannappel, 2010; Huff et al., 2001).

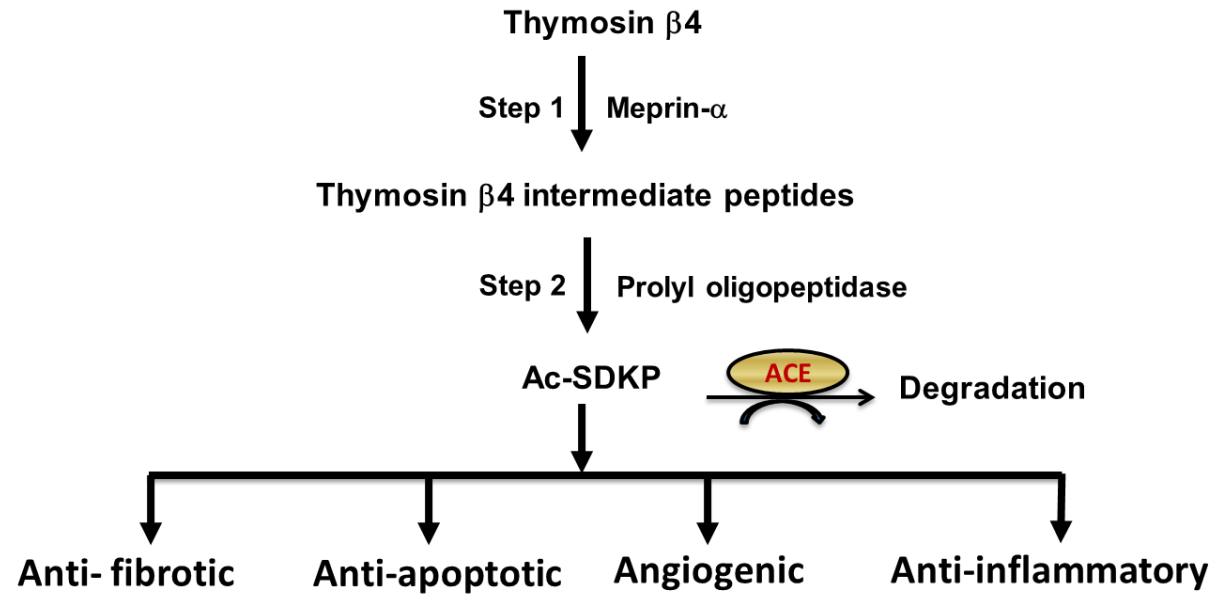

Figure 2. Synthesis and Metabolism of Ac-SDKP © Mani Maheshwari, 2018.

Relationship Between ACE and Ac-SDKP Content in Different Organs

Though Ac-SDKP is reported to be present in kidney and heart, the highest concentration of Ac-SDKP is found in lymphoid organs such as spleen and thymus. Interestingly, Ac-SDKP precursor Tβ4 is also present in relatively high concentration in these lymphoid organs (Pradelles, Frobert, Creminon, Ivonine, & Frindel, 1991). The two major regulators of Ac-SDKP concentration in tissues are 1) presence of its precursor and the enzymes that degrade it, and 2) the amount of ACE that degrades it. Studies reported that tissues such as lungs have a relatively higher Tβ4 per milligram of the tissue, compared to thymus and bone marrow; however, Ac-SDKP concentration is found to be lower in the lungs due to higher ACE activity (Pradelles et al., 1991). Similarly, lymphoid organs such as spleen, thymus, and bone marrow have either very low ACE activity or ACE is absent in these organs and thus have higher Ac-SDKP (Junot et al., 1999). Testis has higher levels of Ac-SDKP compared to other tissues and it is because of lack of N terminal domain of ACE in testis (Stephan et al., 2000). It is well known that lack of N terminal domain of ACE is involved in the degradation of Ac-SDKP (Kanasaki et al., 2014).

Properties of Ac-SDKP

A Anti-Fibrotic Effects of Ac-SDKP

In physiological conditions, fibrosis is defined as the formation of excess connective tissue and it is a mechanism involved in wound healing and tissue repair, but in pathological conditions, there is an accumulation of extracellular matrix (ECM) proteins that leads to the thickening of the affected tissue and eventually to tissue damage. Ac-SDKP has been shown to have anti-fibrotic effects in various experimental models. Ac-SDKP prevents mesangial matrix expansion in diabetic db/db mice (Nitta et al., 2016). Ac-SDKP has been shown to reduce renal interstitial fibrosis in Dahl salt sensitive rats (Worou et al., 2015). Fibroblasts play a major role

in fibrosis; Ac-SDKP has been shown to suppress the proliferation of renal and cardiac fibroblasts (Rhaleb, Peng, Harding, et al., 2001).

Accumulation of pro-fibrotic cytokines around the kidney results in the ECM-producing cell activation responsible for renal fibrosis. Fibroblasts are the major matrix producing cells and are a source of fibronectin, Type l and Type lll collagen. Activated fibroblasts are an important source for the production of extracellular matrix but almost all cell types are responsible for ECM production. ECM includes resident fibroblasts, vascular smooth muscle cells, tubular epithelial cells, and macrophages (Strutz & Zeisberg, 2006). Transforming Growth Factor beta (TGFβ) is a profibrotic cytokine and is important in ECM production. Blocking either TGFβ or the TGFβ stimulated Smad transcriptional factor has been shown to have anti-fibrotic effects (Inagaki & Okazaki, 2007). In fibrotic kidneys, fibroblasts expressing alpha smooth muscle actin (α-SMA) are called myofibroblasts which possess unique contractile properties; they play a role in renal fibrosis (Grande & Lopez-Novoa, 2009).

Ac-SDKP treatment has been shown to ameliorate renal fibrosis and glomerulosclerosis in hypertensive rats and in various other diabetic and non-diabetic models without having an effect on blood pressure. Morel et al. have shown that Ac-SDKP reduced the high salt-induced interstitial fibrosis and glomerulosclerosis in Dahl salt sensitive rats (Worou et al., 2015). α-SMA has been shown to decrease with Ac-SDKP treatment (H. Xu et al., 2012). In vitro experiments with activated fibroblasts have also shown the anti-fibrotic effects of Ac-SDKP. The study done by Peng et al. demonstrated that human cardiac fibroblasts treated with TGFβ transformed into myofibroblasts as indicated by the increased expression of α-SMA and the embryonic form of smooth muscle myosin when compared to untreated cells (Peng, Carretero, Peterson, & Rhaleb, 2010). This study also found that Ac-SDKP administration stopped the

TGFβ induced differentiation of cardiac fibroblasts into myofibroblasts. Our lab along with other investigators demonstrated that Ac-SDKP shows its anti-fibrotic activity mainly by inhibiting Smad 2 phosphorylation; the anti-TGFβ/Smad pathway is a key to understand the anti-fibrotic effect of Ac-SDKP (Border & Noble, 1994; Peng et al., 2010; Pokharel et al., 2002).

B Anti-Inflammatory Effects of Ac-SDKP

Inflammation is required for tissue repair and is closely linked with regeneration of parenchyma cells that fill the tissue defects with fibrous tissue leading to scar formation (Wynn, 2007). However, in conditions where fibrosis is progressive with sustained inflammation, there is an abnormal wound healing (Y. Liu, 2011). In various experimental models, Ac-SDKP has shown to reduce inflammation in the heart and kidney ameliorating tissue fibrosis (Omata et al., 2006; Peng et al., 2007). Rhaleb et al. have shown the anti-inflammatory effect of Ac-SDKP by decreasing the inflammatory cell infiltration in deoxycorticosterone acetate-salt hypertensive mice (Rhaleb et al., 2011). Although the mechanism by which Ac-SDKP acts as an anti-inflammatory is not very clear, it may be due to inhibition of monocyte chemoattractant protein-1 (MCP-1). MCP-1 is one of the key chemokines involved in the regulation of macrophage infiltration, and Ac-SDKP has been shown to suppress MCP-1 (M. Wang, Liu, Jia, Mu, & Xie, 2010). In addition to MCP-1, Ac-SDKP has also been shown to inhibit nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), a key pro-inflammatory transcriptional factor, along with other associated chemokines (Nakagawa et al., 2012).

C Angiogenic Effects of Ac-SDKP

Ac-SDKP is known to stimulate the cell growth and proliferation of vascular endothelial cells, and thus promotes angiogenesis both in vivo and in vitro (J. M. Liu et al., 2003). The beneficial effects of Ac-SDKP seen in myocardial infarction, hind limb ischemia-reperfusion and

stroke may also be due to its pro-angiogenic activity by allowing new blood vessel generation and supplying oxygen and nutrients to the damaged tissue (F. Yang et al., 2004; L. Zhang et al., 2014). Liu et al. demonstrated that Ac-SDKP stimulated in-vitro endothelial cell migration and differentiation into capillary-like structures and increased vascular density of abdominal muscles (J. M. Liu et al., 2003).

D Anti-Apoptotic Effects of Ac-SDKP

Apoptosis is defined as a programmed cell death. Increased amount of apoptosis is associated with fibrosis of the tissues and inhibition of apoptotic pathways is linked with inhibition of fibrosis in various organs (Dooley, Harvey, & Thomas, 2011). Originally, Ac-SDKP was identified as a regulator of stem cells. Studies have shown that Ac-SDKP can suppress apoptosis of hematopoietic cells induced by stress like chemotherapy, radiation, and high temperature (Bogden et al., 1991; Watanabe et al., 1996).

Ac-SDKP and Its Renoprotective Effects

A Hypertension

About 75 million US adults (32%) are hypertensive and more than 50% of hypertensive patients are salt sensitive (Weinberger, Fineberg, Fineberg, & Weinberger, 2001). Salt sensitive individuals have increased blood pressure on sodium intake and their pressure natriuresis curve shifts to the right (J. E. Hall, Mizelle, Hildebrandt, & Brands, 1990). Salt-sensitive hypertension is highly prevalent in African-Americans who develop end-stage renal disease at a three times higher rate than Caucasians (Whelton et al., 2016). Hypertension is one of the major causes of end stage renal disease. But, only approximately 20% of these hypertensive patients respond well with anti-hypertensive medications (Whelton et al., 2016). Therefore there is an urgent need for novel effective therapies that reduce the target organ injury caused by high blood pressure. In

deoxycorticosterone acetate (DOCA) salt form of hypertension in rats and mice, elevated blood pressure for 6-8 weeks caused significant renal fibrosis and damage. Ac-SDKP ameliorated renal fibrosis by reducing collagen synthesis without affecting the blood pressure (Peng et al., 2001). In DOCA-salt hypertension in mice, Ac-SDKP reduced urinary albuminuria by increasing nephrin expression in the kidney. Nephrin is a key protein that forms the integral part of the glomerular filtration barrier in the kidney (Rhaleb et al., 2011). Low expression of renal nephrin is associated with increased urinary albuminuria, which is an early marker for renal damage. Additionally, Morel et al. have shown in Dahl salt-sensitive rats that treatment with Ac-SDKP ameliorated renal injury by reducing urinary albuminuria, fibrosis and infiltration of macrophages and T-cells in the kidney (Worou et al., 2015).

B Renal Diseases

In the chronic kidney disease model generated by 5/6 nephrectomy in rats, Ac-SDKP treatment not only prevented but even reversed the tissue damage in the kidney. In 5/6 nephrectomy, Ac-SDKP reduced urinary albumin by increasing renal nephrin content and improved renal function by restoring glomerular filtration rate (GFR). In addition, Ac-SDKP reduced the renal collagen content, glomerulosclerosis, and infiltration of macrophages (Liao et al., 2010). In the unilateral ureter obstruction (UUO) model in rodents, Ac-SDKP treatment for two weeks in Wistar rats significantly attenuated renal interstitial inflammation and fibrosis (M. Wang et al., 2010). In UUO C57BL/6 mice, Ac-SDKP treatment reduced renal fibrosis in the early and late phases by decreasing profibrotic plasminogen activator inhibitor-1 expression. Similar renoprotection was observed in UUO BALB/C genetic background mice, where Ac-SDKP partly mediated the anti-fibrotic effect of captopril (Chan et al., 2018). Munich Wistar Fromter (MWF) rats develop spontaneous progressive nephropathy. In these rats, microRNA

(MiR)-324-3p was the most upregulated MiR in the microdissected glomeruli and POP was found to be the target of MiR-324-3p (Macconi et al., 2012). ACE inhibitor treatment attenuated the renal fibrosis in MWF rats which was accompanied by the downregulation of MiR-324-3p. Decreased MiR-324-3p expression was associated with increased POP activity (Macconi et al., 2012). In this study, high plasma and urinary Ac-SDKP content following ACE inhibition was suggested to be the combined result of 1) blockade in endogenous Ac-SDKP degradation by inhibiting ACE, and 2) increased endogenous Ac-SDKP synthesis by increased POP activity (Macconi et al., 2012).

C Diabetes

Diabetic nephropathy is one of the leading causes of end-stage renal disease worldwide (Ghaderian, Hayati, Shayanpour, & Beladi Mousavi, 2015). ACE inhibitor therapy is usually prescribed in this condition. In *db/db* mice, Ac-SDKP treatment showed renoprotection by preventing mesangial matrix expansion and reduced expression of pro-fibrotic molecules in the TGFβ/Smad signaling pathway (Shibuya et al., 2005). In streptozotocin (STZ) induced type-1 diabetic rats, Ac-SDKP treatment improved renal fibrosis by decreasing renal interstitial and perivascular fibrosis (Castoldi et al., 2013). Endothelial-mesenchymal transition (EndMT) has emerged as an important source of collagen producing myofibroblasts and may contribute to the progression of renal fibrosis. STZ induced diabetes in CD-1 mice showed lower amount of urinary Ac-SDKP, compared to non-diabetic control mice (Nagai et al., 2014). Ac-SDKP treatment reduced EndMT in the kidney of the diabetic animals. These effects were associated with an upregulation of anti-fibrotic MiR let-7b and fibroblast growth factor receptor (Nagai et al., 2014). In a different study involving mice with type-1 diabetes (STZ-induced) and type-2 diabetes (*db/db*), oral administration of Ac-SDKP showed similar levels of renal anti-fibrotic

effects by upregulating anti-fibrotic let-7b and MiR-29 levels (Nitta et al., 2016). Ac-SDKP beneficial effects in STZ-induced diabetic mice were proposed to be the result of a crosstalk between anti-fibrotic microRNAs involving the let-7b/MiR-29 axis (Srivastava et al., 2016).

D Autoimmune Disorder

Systemic lupus erythematosus (SLE) is an autoimmune disorder with excessive renal inflammation. It is characterized by deposition of anti-nuclear antibodies along the glomerular basement membrane resulting in glomerulonephritis and possible development of renal failure. MRL/lpr lupus mice mimic the human form of SLE and are used extensively to study the mechanisms responsible for renal inflammation in autoimmune diseases. It has been reported that in MRL/lpr lupus mice, Ac-SDKP reduced renal infiltration of macrophages and T-Cells and improved proteinuria (Tan et al., 2012). Ac-SDKP reduced expression of inflammatory markers TNF-α and NF-κb and reduced expression of fibrotic markers TGFβ1, α-SMA, fibronectin, and activated Smad2/3 (Tan et al., 2012). A study by Tang-Dong et al. recently observed that Ac-SDKP renoprotective effects in MRL/lpr were, in part, due to the decreased expression of renal complement system C5/C5a and C5b-9 and reduction in pro-inflammatory intercellular adhesion molecule ICAM-1 in the kidney (Liao et al., 2015). Additionally, Ac-SDKP treatment ameliorated the progression of renal damage and fibrosis by reducing urinary albuminuria, glomerulosclerosis, renal interstitial collagen and infiltration of renal ED-1 positive macrophages in a rat model of nephritis generated by administration of anti-glomerular basement membrane antibody (Omata et al., 2006).

Ac-SDKP and Its Cardioprotective Effects

A Heart Failure and Dysfunction

One of the most common causes of the heart failure is atherosclerosis and it is the leading cause of deaths worldwide (Low Wang, Hess, Hiatt, & Goldfine, 2016). In atherosclerosis, the plaque (made up of fat, cholesterol, calcium and other substances formed in blood) is formed inside the arteries and potentially limit the blood-supply to the coronary circulation. The other causes of heart failure or heart damage may result from pre-existing comorbidities including high blood pressure, diabetes, obesity, viral infection, environmental, and genetic factors. It has been shown that in most of the models of cardiac damage, infiltration of lymphocytes, monocytes and neutrophils marks the first steps that are essential for the tissue repair, fibrosis and remodeling. Ac-SDKP has been shown to have protective effects in various models of cardiac damage. For example, in a myocardial infarction model in Sprague Dawley rats, Ac-SDKP treatment prevented and reversed inflammation in the non-infarcted area of the left ventricle primarily by decreasing macrophages and TGFβ (Rasoul et al., 2004). In a different study, combination treatment of Ac-SDKP with the stem cell homing factor and stromal derived factor-1 applied directly at the border zone of the infarcted area initiated at 4 weeks after myocardial infarction in rats, resulted in decreased infarct size, improved cardiac function, and increased angiogenesis (Song et al., 2014).

In 2-kidney 1-clip and DOCA-salt hypertensive rat models, Ac-SDKP prevented and reversed collagen deposition in the left ventricle (LV) by limiting cardiac fibroblasts proliferation and infiltration of monocytes/macrophages (Peng, Carretero, Brigstock, Oja-Tebbe, & Rhaleb, 2003; Peng et al., 2001; Rhaleb, Peng, Yang, et al., 2001). In Ang-II and DOCA-salt hypertension, Ac-SDKP reduced LV collagen deposition by decreasing TGFβ and p-Smad2 and

these protective effects were blocked by a neutralizing antibody against Ac-SDKP (Peng et al., 2007; Peng et al., 2005). Cavasin et al. found that blocking the Ac-SDKP synthesis by the specific POP inhibitor S17092 exacerbated collagen deposition in the heart suggesting that basal Ac-SDKP is required in preventing excessive fibrosis (Cavasin et al., 2007). Ac-SDKP inhibited cardiac collagen deposition not only by limiting cardiac fibroblast proliferation but also by reducing differentiation of fibroblast into myofibroblasts (Peng et al., 2010).

Diabetes related heart damage imposes a major health burden globally (Bhutani & Bhutani, 2014; Kania, Blyszczuk, & Eriksson, 2009). In STZ-induced diabetic cardiomyopathy in rats, Ac-SDKP reduced interstitial and perivascular cardiac fibrosis and also improved diastolic function without changes in systolic function (Castoldi et al., 2009).

B Myocarditis

Myocarditis represents the cardiac inflammation and injury, which often result from infections with viruses, such as adenoviruses or parvovirus, bacterial infection, toxins or autoimmune disorders (Nakagawa et al., 2012). Myocarditis causes immune cells to recognize the body's own heart cells as antigens and elicit an uncontrolled autoimmune response that results in dilated cardiomyopathy and heart failure (Kania et al., 2009). Immune cells such as neutrophils, monocytes and lymphocytes infiltrate the myocardium and initiate an early immune response, which is followed by the resolution of inflammation and cardiac fibrosis (Frangogiannis, 2012). In normal homeostasis, a balance is maintained between collagen synthesis and its degradation. Degradation of collagen is tightly controlled by the zinc proteases known as matrix metalloproteinase (MMP) 2 and MMP-9 and their physiological tissue inhibitors tissue inhibitor of metalloproteinases-1 (TIMP)-1 and TIMP-2. A dysregulation of MMPs and TIMPs often results in accumulation of collagen and tissue fibrosis (Kania et al.,

2009). In experimentally induced myocarditis in rats, Ac-SDKP reduced the infiltration of macrophages, T-cells, and reduced the expression of pro-inflammatory interleukin (IL) -1β and IL-17. Ac-SDKP also reduced cardiac hypertrophy and improved cardiac function (Nakagawa et al., 2012).

Ac-SDKP and Its Neuroprotective Effects

Ac-SDKP has also been shown to have beneficial effects in conditions like stroke. Stroke is one of the leading causes of deaths worldwide (L. Zhang et al., 2014). Almost 80% of stroke cases are due to cerebral arterial thrombosis (Ding et al., 2014). In clinics, the only treatment available to treat cerebral arterial thrombosis is the administration of tissue plasminogen activator (tPA). The drawback of using tPA is the risk of brain hemorrhage (Ding et al., 2014). In a study performed in Wistar rats subjected to embolic stroke, MRI data showed that the combined treatment of Ac-SDKP and tPA initiated at four hours significantly reduced the blood brain barrier (BBB) leakage and reduced ischemic lesions compared to the monotherapy of tPA (L. Zhang et al., 2014). Inactivation of NF-κb and TGFβ signaling pathway in the brain by Ac-SDKP are the reasons behind its neuroprotective effects. In a study of traumatic brain injury (TBI) induced by controlled cortical impact, Ac-SDKP treatment initiated at one hour significantly improved sensorimotor function and spatial learning. Ac-SDKP reduced activation of microglia/macrophages, reduced fibrin accumulation, and enhanced neurogenesis (Y. Zhang et al., 2017). However, the mechanism of neuroprotection of Ac-SDKP in stroke and brain injury is not clear. Further studies are required to elucidate the mechanisms underlying the neuroprotective effects of Ac-SDKP.

Table 1. Summary of the Anti-Fibrotic and Anti-Inflammatory Effects Mediated by Ac-SDKP in Kidneys of Various Rat/Mouse Models

Model	Effect	Reference
Ang II Induced Hypertension	Lowered fibrosis and inflammation	(Peng et al., 2007)
Aldosterone Induced Hypertension	Lowered fibrosis	(Peng et al., 2001)
DOCA Salt Induced Hypertension	Lowered fibrosis and inflammation, reduced albuminuria	(Rhaleb et al., 2011)
Dahl Salt Sensitive Hypertension	Lowered fibrosis and inflammation, reduced albuminuria	(Worou et al., 2015)
5/6 Nephrectomy Induced Hypertension	Lowered fibrosis and inflammation, reduced albuminuria, improved GFR	(Liao et al., 2010)
Lupus Nephritis	Lowered fibrosis and inflammation, reduced proteinuria, improved renal function	(Tan et al., 2012)
Diabetic Nephropathy	Lowered glomerulosclerosis, inhibited endothelial to mesenchymal transition	(Nagai et al., 2014)
Obesity Induced Hypertension (Zucker Obese Rats)	Lowered fibrosis, inflammation, reduced hypertension.	(Maheshwari et al., 2018)

Prevalence of Obesity

Obesity is considered by the World Health Organization to be a major threat to global health. Obesity is associated with diabetes and hypertension which are the two major causes for end stage renal disease (M. E. Hall et al., 2014). More than two-thirds (75%) of the US population is considered overweight or obese and of these one-third (35%) are obese with a body mass index of more than 30kg/m^2 (J. E. Hall et al., 2015). In spite of increased awareness, the prevalence of obesity has continued to rise and it poses a worldwide problem. Various factors are associated with obesity, in particular an increase in the per capita food supplies and consumption, particularly high calorie foods (Hurt, Kulisek, Buchanan, & McClave, 2010). Additionally, lack of physical activity and sedentary lifestyle contribute to the cause

(Duvigneaud et al., 2007). However, the genetic component associated with obesity cannot be ignored. Everyone living in an urban setting is not obese, suggesting that there is an underlying genetic mechanism operating at an individual level. Most likely, the genes participating in determining energy balance, metabolism and some behavioral traits cooperate with environmental changes to either regulate, or fail to regulate, weight gain (Hill, Wyatt, & Peters, 2012).

There are various ways to define obesity. Currently, the most widely accepted standard is Body Mass Index (BMI). BMI is defined as weight in kg divided by height in meters, squared. Individuals having BMI above 25 are considered overweight and are at risk for cardiovascular and renal damage. Nearly two thirds of the US population (66%) is overweight by this definition (Flegal, Kruszon-Moran, Carroll, Fryar, & Ogden, 2016). It is accepted that this method has limitations, for instance excess fat is not always present with a moderately increased BMI. In contrast, a person with normal BMI might not always be protected from the sequelae of obesity (Coutinho et al., 2011). Another limitation is that BMI does not differentiate between upper body fat and lower body fat (Yusuf et al., 2005). Most of the studies have shown that the upper body fat is correlated with cardiovascular risks and death compared to lower body fat (Gurunathan & Myles, 2016). The National Heart, Lung, and Blood Institute considers three key measures to assess obesity and health risks: waist circumference, BMI and risk factors for diseases and conditions associated with obesity. The waist to hip ratio (WHR) which is an index of upper body, tells more about hypertension and cardiovascular diseases compared to other available methods to define obesity (Yusuf et al., 2005). No matter how obesity is defined, there is enough evidence to indicate that excessive body weight is associated with increased health problems and mortality rates. The magnitude of this obesity-associated risk is further influenced by age,

gender, and the fat distribution pattern. For example, abdominal obesity appears to disproportionately increase the risk of developing certain comorbidities like cardiovascular disease and end stage kidney damage (Cornier et al., 2011; Despres, 2012).

Obesity and Salt Sensitive Hypertension

In parallel to obesity, the prevalence of hypertension has also increased in the last decade. Both obesity and hypertension are major health issues in the United States (Saydah et al., 2014). Approximately 30% of hypertension cases are attributed to obesity (Dornfeld, Maxwell, Waks, Schroth, & Tuck, 1985). The rise in the prevalence of obesity is a chief factor in the increased incidence of hypertension, which is a major risk factor for heart disease, stroke, kidney failure, and other serious health complications. The incidence of both obesity and hypertension is more common in developed countries but the co-occurrence of these two factors in the same individual cannot be explained by a mere random coincidence. According to the risk estimates from the Framingham Heart Study, obesity is linked to about 75% of male and 65% of female cases of hypertension (Wilson, D'Agostino, Sullivan, Parise, & Kannel, 2002). Indicators for the risk of hypertension include obesity, abdominal fat and weight gain. There is enough evidence to show that some individuals can excrete an excess dietary salt intake without an increase in blood pressure while others cannot excrete excess dietary salt intake without increasing blood pressure (Choi, Park, & Ha, 2015). Salt sensitivity is arbitrarily defined as the 10% increase in the blood pressure when an individual is challenged with high salt diet than that with low salt diet (Burnier, Wuerzner, & Bochud, 2015). Kawasaki et al. and later on Weinberger et al. were among the first to recognize the heterogeneity of the blood pressure response to salt and to develop the concept of salt sensitivity and salt resistance in humans (Kawasaki, Delea, Bartter, & Smith, 1978; Weinberger, Miller, Luft, Grim, & Fineberg, 1986).

Genetic factors such as ethnicity also play a role in the incidences of obesity related hypertension. African-American adults are nearly 1.5 times as likely to be obese compared with white adults ("National Center for Health Statistics. Health, United States, 2016: With Chartbook on Long-term Trends in Health. Hyattsville, Maryland. 2016"). Approximately 47.8 percent of African-Americans are obese (including 37.1 percent of men and 56.6 percent of women) compared with 32.6 percent of whites (including 32.4 percent of men and 32.8 percent of women) (Svetkey, McKeown, & Wilson, 1996). More than 75% of African Americans are overweight or obese (including 69 percent of men and 82.0 percent of women) compared with 67.2 percent of whites (including 71.4 percent of men and 63.2 percent of women) (Svetkey et al., 1996). The incidence of hypertension goes in parallel with obesity. More than 40 percent of non-Hispanic African-American men and women have high blood pressure. Salt sensitivity is more prevalent in African-Americans compared to Caucasians. 73% of all African-American hypertensive patients are salt sensitive (Peters & Flack, 2000; Svetkey et al., 1996).

Mechanisms of Salt Sensitive Hypertension

A Renal Determinants of Salt Sensitivity

In the past much work has been done to elucidate the importance of the kidney in the pathogenesis of hypertension. In the 1970s, animal experiments performed by Dahl suggested that when the kidney from a salt sensitive rat was transplanted in a salt resistant rat, the recipient developed hypertension and vice versa suggesting the role of the kidney in the pathogenesis of hypertension (Rettig & Grisk, 2005). It was believed that hypertension associated with high salt intake is due to the defect in the renal excretion of sodium. Guyton described the pressure natriuresis curve first in dogs which reflect the relationship between the salt balance and systemic blood pressure in normal and pathological conditions (Guyton, 1989). Pressure

natriuresis is defined as the relationship between sodium excretion and mean arterial pressure. In normal conditions, an individual requires a certain systemic blood pressure to eliminate sodium in order to maintain normal sodium homeostasis but in salt sensitive subjects, this pressure natriuresis curve shifts to the right, which indicates that the salt sensitive individuals require higher systemic pressure to excrete sodium in order to maintain sodium homeostasis. According to his hypothesis, whatever the reason for hypertension, the pressure natriuresis curve is always affected. Impaired pressure natriuresis curve implies the importance of sodium and water excretion by the kidneys in regulating the blood pressure. Later Kimura and Brenner proposed three major mechanisms which cause hypertension: increased pre glomerular vascular resistance, a decrease in whole kidney ultrafiltration and an increase in tubular sodium reabsorption (Kimura & Brenner, 1993). According to them, preglomerular vascular resistance cause hypertension in salt resistance and an alteration in renal sodium handling and the loss of nephron mass is responsible for hypertension in salt sensitive individuals. In recent years, Johnson and Schreiner have described the role of microvascular injury and tubulointerstitial fibrosis in the development of hypertension via shifting the pressure natriuresis curve to the right (Johnson & Schreiner, 1997). It has been shown that infusions of Ang II and phenylephrine cause microvascular injury and thus the hypertension without the sympathetic or renin angiotensin system activity (Johnson et al., 1999; Lombardi et al., 1999). Thus, these hypotheses along with animal experiments done in previous years suggest the role of the kidney in the pathogenesis of salt sensitive hypertension.

B Sodium Retaining Mechanisms

Adrenal and Sympathetic Nervous System: Increased activity of the sympathetic nervous system leads to increased sodium retention and decreased sodium excretion (Tuck, 1986). Salt

sensitive individuals with essential hypertension showed an abnormal relationship between sodium excretion and plasma noradrenaline levels (Campese et al., 1982). In normal and salt resistant individuals, a high sodium diet leads to decreased plasma concentrations of noradrenaline but in salt sensitive individuals, a high salt diet is accompanied by a rise, no change or a decrease in plasma concentrations of noradrenaline (Campese et al., 1982). Obese individuals are often associated with both salt sensitive hypertension and increased SNS activity. The three major mechanisms, which are associated with the anti-natriuretic effect of increased sympathetic nervous system activity, are: - increased renin secretion, reduced renal blood flow, and increased renal tubular reabsorption (Fujita, 2014).

With-no-lysine kinase-4 (WNK-4), a serine threonine kinase, is a negative regulator of the thiazide-sensitive sodium chloride cotransporter (NCC) (C. L. Yang, Angell, Mitchell, & Ellison, 2003; Zhou et al., 2012). Normally, WNK-4 inhibits NCC activity leading to a decrease in sodium reabsorption in distal convoluted tubule to maintain normal blood pressure (Lalioti et al., 2006). Various reports showed that the dietary sodium intake modulates the expression of WNK kinases and in turn affects NCC activity (Mu et al., 2011; O'Reilly et al., 2006). Excessive salt intake in salt sensitive individuals causes an increase renal sympathetic activity. Ang II and aldosterone are believed to be involved in switching WNK-4 to a functional state thereby promoting NCC activation (Figure 3) (Mu et al., 2011). Ang II acts in a SPS-1 related proline/ alanine-rich kinase (SPAK) dependent manner (Castaneda-Bueno et al., 2012) and aldosterone activates NCC via either the WNK-4-SPAK- dependent or the WNK-4-extracellular signal-regulated kinase1/2 (ERK ½) signaling pathway (Ko et al., 2013; Lai et al., 2012).

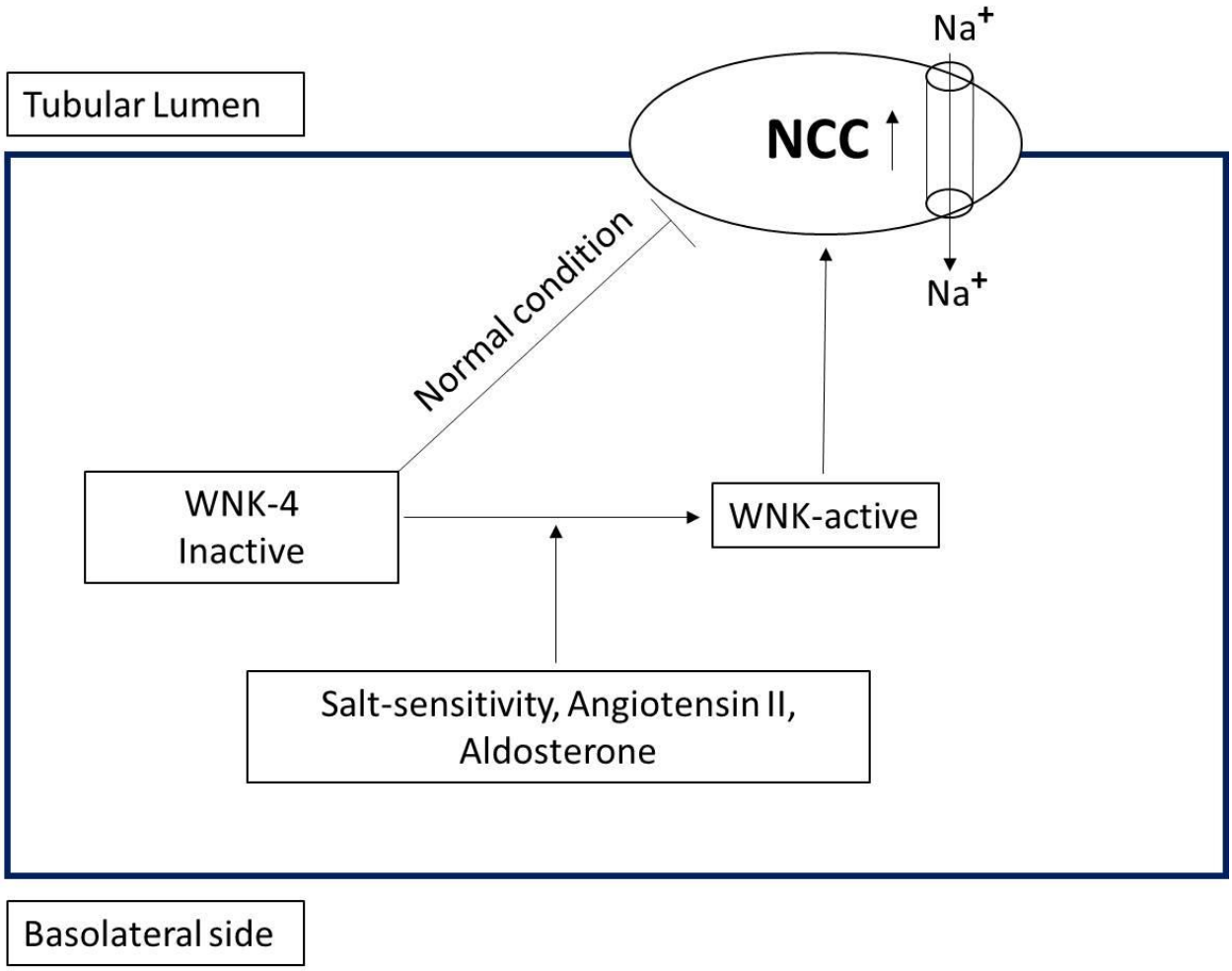

Figure 3. Regulation of NCC Transporter by WNK kinase in Distal Tubule Segment of the Nephron © Mani Maheshwari, 2018.

The adrenal system is also involved in the salt sensitive hypertension. Normally levels of plasma aldosterone are counterbalanced by dietary salt intake by changes in the levels of circulating Ang II. Increased salt intake with a continuous infusion of aldosterone increases blood pressure and proteinuria seen in primary aldosteronism. On a low salt diet, aldosterone induced hypertension and renal injury do not occur which tells us that salt is indispensable for aldosterone induced mineralocorticoid receptor (MR) activation and eventually leads to hypertension (Fujita, 2014; Shibata et al., 2011). Studies have shown that in obese hypertensive rats, the negative feedback regulation of aldosterone secretion by salt is impaired, which leads to

salt sensitive hypertension and renal damage through MR activation (Fujita, 2010). In Dahl salt sensitive rats, salt loading inhibits the glucocorticoid-inducible-kinase 1 (SGK1) which is a downstream mediator of MRs in spite of the appropriate suppression of aldosterone. Salt loading mediated SGK1 inhibition despite lower levels of aldosterone suggests that MRs are activated in an aldosterone independent manner. Rac 1, a member of Rho-guanine triphosphate hydroxylases family, has been shown to be involved in aldosterone independent MR activation (Aoi, Niisato, Sawabe, Miyazaki, & Marunaka, 2006; Farjah, Roxas, Geenen, & Danziger, 2003).

C Hyperinsulinemia and/or Insulin Resistance

Insulin resistance is defined as the inability of the cells to respond normally to the insulin hormone. Obesity leads to insulin resistance and is often accompanied by hyperinsulinemia. African Americans are more salt sensitive and hyperinsulinemic when compared to the white population (Sanada, Jones, & Jose, 2011). Normally, insulin has a sodium retaining effect and it is because of its direct action on the renal tubules. Sodium retention due to hyperinsulinemia could lead to a rise in blood pressure. Insulin is also known to have an acute sympathoexcitatory effect and it has depressor effect of peripheral vasodilation (Mendizabal, Llorens, & Nava, 2013).

D Renin Angiotensin System

The renin angiotensin system (RAS) plays a very important role in controlling body fluid volume, electrolyte balance, and blood pressure. RAS is a key factor in many cases of essential hypertension and it mediates its action via Ang II (Yim & Yoo, 2008). Various studies have shown ACE inhibitors and Ang II blockers are used in controlling essential hypertension (Baltatzi, Savopoulos, & Hatzitolios, 2011). Renin is the rate-limiting enzyme in Ang II formation. When the plasma sodium concentration is lower than the normal or there is decreased

renal perfusion, the juxtaglomerular cells in the kidney sense that and convert pro renin to renin which enters in the circulation. Plasma renin then converts angiotensinogen, a glycoprotein consisting of 429 amino acids produced by hepatocytes in the liver, to the decapeptide Angiotensin I. Angiotensin I gets converted to Ang II, an octapeptide, by angiotensin converting enzyme (ACE) which is found on the endothelial cells of the capillaries throughout the body, within the lungs and the epithelial cells of the kidney (Ichihara, Kobori, Nishiyama, & Navar, 2004). Ang II is a potent vasoconstrictor, which causes the constriction of the arterioles leading to an increased arterial blood pressure. Ang II exerts its effect via acting on its receptors, Angiotensin 1 (AT1) and Angiotensin 2 (AT2). AT 2 receptors counteract the vasoconstrictor and growth stimulatory action of AT1 receptors (Yim & Yoo, 2008).

A high salt diet suppresses Ang II levels through physiological blood pressure control mechanisms. In 40-50% cases of essential hypertension, the adrenal and renal vascular responses to Ang II are not as expected with the salt intake. They are referred to as "nonmodulators" (Williams & Hollenberg, 1989). In salt sensitive individuals there are several structural alterations in the genes, which code for various components of RAS. Poch et al. evaluated the association between the genetic polymorphisms of the RAS and salt sensitive hypertension in humans (Poch et al., 2001). As it is known, salt sensitivity varies with race. African-Americans are more prone to develop hypertension compared to Caucasians. It is shown that the RAS of African-Americans is more salt sensitive and they develop hypertension with less intake of salt compared to the Caucasian population (Luft et al., 1991; Richardson & Piepho, 2000).

E Oxidative Stress and Renal Infiltration of Immune Cells

In both experimental and human hypertension, oxidative stress has been shown to play a role. Reactive oxygen species (ROS) play a critical role in the development of hypertension and

there is evidence showing hypertension leads to the generation of ROS creating a vicious cycle (S. Xu & Touyz, 2006). In various models of hypertension such as Ang II induced hypertension, DOCA salt hypertension, and spontaneously hypertensive rats (SHRs), stimulation of NAD(P)H oxidase is the primary source of the generation of oxidants (Fukui et al., 1997; Landmesser & Harrison, 2001; Zalba et al., 2000). In patients with essential hypertension, NAD(P)H is the main source of superoxide production in the vascular smooth muscles (Lassegue & Clempus, 2003). ROS can inactivate nitric oxide production resulting in the loss of vasodilation. Depending on the amount produced and the vascular bed, ROS can exert vasodilatory or vasoconstrictory effects but mainly it has vasoconstrictor effects. The vasoconstrictory effect of ROS is mainly due to the generation of vasoconstrictive eicosanoids like prostaglandin $F_{2\alpha}$ from the oxidation of arachidonic acid and also the inhibition of synthesis of vasodilatory eicosanoids such as Prostaglandin I2 (Korbecki, Baranowska-Bosiacka, Gutowska, & Chlubek, 2013). In addition to the systemic effects, recent studies suggested that oxidative stress in the kidney is involved in the pathophysiology of the sodium retention because it leads to the tubulointerstitial accumulation of Ang II-positive cells (Imig & Ryan, 2013; Majid, Prieto, & Navar, 2015).

F Tubulointerstitial Inflammation and Hypertension

There is evidence that the immune cells infiltrating in the kidney leads to the sodium retention and thus contribute to the hypertension (Wade, Abais-Battad, & Mattson, 2016). Tubulointerstitial infiltration of macrophages and lymphocytes is present in almost all the experimental models of salt sensitive hypertension such as DOCA-salt hypertension, post-Ang II infusion salt-sensitive hypertension, hyperuricemia-induced hypertension, two-kidney one- clip hypertension, as well as genetic models of hypertension such as SHR and the double transgenic rat harboring the human renin and angiotensinogen genes (Rodriguez-Iturbe, Quiroz, Kim, &

Vaziri, 2005; Rodriguez-Iturbe, Vaziri, Herrera-Acosta, & Johnson, 2004; Rodriguez-Iturbe, Zhan, Quiroz, Sindhu, & Vaziri, 2003; Tapia et al., 2003). The mechanism by which immune cell infiltration leads to hypertension is not clear, but it is speculated that the accumulation of immune cells leads to the intrarenal Ang II, and Ang II possesses sodium retaining effects. In experimental models of hypertension, it has been shown that infiltrating T cells and macrophages express Ang II. Interstitial accumulation of Ang II positive cells has been postulated as a primary reason for the sodium retention in patients with nephrotic syndrome. Apart from sodium retaining effects of Ang II, there are other potential consequences of Ang II activity in the kidney such as the activation of transcription factors and a signaling cascade which may stimulate superoxide production mediated by NAD(P)H oxidase (Sonnenberg, Honrath, Chong, & Wilson, 1986). In models of salt sensitive hypertension, interstitial inflammation is associated with increased apoptosis and activation of NF-κB. Inhibition of NF-κB reduces the accumulation of inflammatory cells. Several mechanisms that regulate cellular ion transport have been evaluated in hypertensive patients. According to the classical hypothesis, hypertension in salt sensitive individuals might be due to an impaired renal function, which leads to an increased Na^+ reabsorption and reduced Na^+ excretion. The sodium retention leads to volume expansion and subsequently the secretion of an ouabain-like substance. This ouabain-like substance inhibits Na^+, K^+ ATPase in the kidney and maintains Na^+ balance, although at higher levels. The inhibition of Na^+, K^+ ATPase activity in vascular smooth muscle cells and brain leads to hypertension (Haddy, 1987). The role of Na^+, K^+ ATPase in hypertension is supported by the evidence that long term administration of ouabain induced hypertension in normal rats (Yuan et al., 1993). Some other studies have shown contradicting results with circulating levels of this

ouabain-like compound (J. Wang, Tempini, Schnyder, & Montani, 1999). There is no conclusive evidence that genetic mutation in the sodium pump subunits are involved in hypertension.

Elevated rates of sodium hydrogen exchanger (NHE) in the cell membrane of blood vessels and renal tubules may play a role in the pathophysiology of hypertension (Li, Shull, Miguel-Qin, Chen, & Zhuo, 2015). Increased activity of NHE might be due to systemic hormonal or metabolic factors (e.g. high Na intake, insulin), to intracellular factors (protein kinase C, calcium calmodulin), or to post-translational modifications (Cingolani & Ennis, 2007). It has been shown that high sodium intake increases calcium in lymphocytes of salt sensitive but not salt resistant hypertensive patients (Alexiewicz et al., 1992). This increase in calcium might alter the activity of NHE exchanger (Baartscheer et al., 2008).

Mechanisms of Progression of Chronic Kidney Disease

A Systemic and Glomerular Hypertension

Hypertension is associated with chronic kidney disease (CKD), and controlling blood pressure is a key in the treatment of CKD (Judd & Calhoun, 2015). The glomerulus has a unique structure with afferent and efferent arteriole working in such a way that in normal conditions, modulation of glomerular perfusion does not affect the systemic blood pressure. Studies have been done on the remnant kidney models to study chronic kidney disease. In 5/6 nephrectomy, there is hyperfiltration, hyperperfusion, hypertrophy, and focal segmental glomerulosclerosis (FSGS) (Shimamura & Morrison, 1975). Other models with initial podocyte injury, namely the puromycin aminonucleoside model of renal disease, show proteinuria and podocyte damage eventually leading to FSGS (Grond, Weening, & Elema, 1984).

Micropuncture studies at single nephron level have demonstrated that single nephron function increases after renal ablation (Hostetter, Olson, Rennke, Venkatachalam, & Brenner,

2001). Increased single nephron function after renal ablation led to the hypothesis that hyperfitration leads to sclerosis setting a vicious cycle of hyperfitration and glomerulosclerosis. Factors that decrease hyperfiltration such as low protein diet, angiotensin converting enzyme inhibitors (ACEis), and lipid lowering agents were effective in ameliorating glomerular sclerosis whereas the factors that increase glomerular capillary pressure such as high protein diet, and glucocorticoids accelerated glomerulosclerosis (Kakinuma et al., 1992).

B Renin-Angiotensin-Aldosterone System

The components of Renin-angiotensin-aldosterone system (RAAS) have been studied extensively in the progression of chronic kidney disease. ACEIs act mainly on the efferent arteriole (Bosma et al., 2006) and cause a decrease in glomerular capillary pressure by dilatation of the efferent arterioles. The dilatation of efferent arteriole is mainly mediated by the inhibition of Ang II and also by an increase in the production of bradykinin which under normal conditions gets degraded by angiotensin converting enzyme (Kon, Fogo, & Ichikawa, 1993). Angiotensin Type I receptor blockers (ARBs) do not increase bradykinin so they are not able to dilate the efferent arteriole or decrease glomerular capillary pressure to the extent of ACEis, but both ACEis and ARBs have been shown to be efficient to reduce the progression of chronic kidney disease (Lewis, Hunsicker, Bain, & Rohde, 1993; MacKinnon et al., 2006).

ARBs leave the AT2 receptor active and thus theoretically can lead to increased AT2 effects by allowing the unbound Ang II to bind to the receptor. The AT2 receptor has an opposite effect to the AT1 receptor. AT2 receptors are vasodilatory and mediate growth inhibition and apoptosis (Stoll et al., 1995; Yamada, Horiuchi, & Dzau, 1996). Apoptosis mainly decreases the injury as the injured cells are removed without the activation of profibrotic cytokines and chemokines. Studies have shown the beneficial effects of the AT2 receptor in transgenic mice

that over express the AT2 receptor; these mice develop less renal injury than the wild type after subtotal nephrectomy (Hashimoto et al., 2004). In clinical studies, combined therapies of ACEis and ARBs have shown a beneficial effect on decreasing proteinuria and improving glomerular filtration rate (GFR). In a large population study of patients with hypertension along with diabetes and microalbuminuria, the combined therapy reduced the blood pressure along with albuminuria much greater than the monotherapy (Mogensen et al., 2000). Additionally, the combined therapy has an anti-fibrotic effect by an augmented bradykinin and AT2 activities and also by decreased urinary TGFβ (Taal & Brenner, 2002). It is possible to have greater RAS inhibition with the combined therapy ACE inhibitors and by inhibition of Ang ll binding to its AT1 receptor. However, suprapharmacological doses of ACE inhibitors could not inhibit the local RAS completely in various experimental models (Nishiyama, Seth, & Navar, 2002). There are reports showing that the plasma of patients receiving ACE inhibitors still have measurable Ang II which suggests that the non ACE dependent Ang II generation by chymotrypsin sensitive generation enzyme occurs in humans (Ahmad et al., 2011). A new area of research includes the development of renin antagonists that could obviate these obstacles to optimal inhibition of the RAAS. Most of the profibrotic effects of RAAS are due to Ang II. It promotes the migration of endothelial and vascular smooth muscle cells, hypertrophy and hyperplasia of smooth muscle cells and mesangial cells (Wolf & Neilson, 1993). All the components of RAAS are present in macrophages, which are another source of Ang II, and they respond to ACEi and ARB. Ang II also induces other growth factors like TGFβ, plasminogen activator inhibitor-1 (PAI-1), platelet-derived growth factor (PDGF), basic fibroblast growth factor (basic FGF). All these growth factors have an impact on fibrosis (Ketteler, Noble, & Border, 1995; Oikawa, Freeman, Lo, Vaughan, & Fogo, 1997).

Recently aldosterone has been shown to promote fibrosis by both genomic and non-genomic actions regardless of its action to increase blood pressure by salt retention (Brown, 2005; Epstein, 2006). Aldosterone aggravates Ang II induction of PAI-1 and also has a direct effect on fibrosis (Brown, 2005). PAI-1 deficiency decreased the aldosterone induced glomerular injury but it could not prevent the cardiac or aortic injury indicating that aldosterone PAI-1 mediated fibrosis is site specific and also species specific (J. Ma et al., 2006).

C Specific Cytokines/Growth Factors

There are various cytokines/growth factors involved in modulating the glomerular and tubulointerstitial fibrosis; they may act at different stages of injury. Altered gene expression of these cytokines occurs in pathophysiological conditions e.g., PDGF, TGFβ, Ang II, basic FGF, endothelin, various chemokines, peroxisome proliferator-activated receptor gamma (PPAR γ), and PAI-1, among others, in progressive renal fibrosis (Fine, Hammerman, & Abboud, 1992; Kashgarian & Sterzel, 1992). Newer approaches with proteomic and array analysis of renal tissue in animal models and human CKD can identify new targets and markers, and also mediators of progression (Schmid, Henger, & Kretzler, 2006; B. J. Xu et al., 2005). Out of various potential molecules of interest, only a few that have been investigated in depth are discussed below.

Increased PAI-1 corresponds to increased cardiovascular disease and kidney fibrosis (Eddy & Fogo, 2006). Conversely, inhibition of Ang II or aldosterone is linked with a decrease in PAI-1 and leads to a decrease in the sclerosis and even regression of the existing kidney fibrosis (Aldigier, Kanjanbuch, Ma, Brown, & Fogo, 2005; Oikawa et al., 1997). Ang II and aldosterone can also induce PAI-1 expression and subsequent fibrosis independent of TGFβ activation (L. J. Ma et al., 2003). Some of the effects of PAI in mediating fibrosis are

independent of its effects on proteolysis. PAI-1 has also been shown to increase cell migration and epithelial-mesenchymal transition (EMT) and thereby increasing fibrosis in inflammatory and interstitial diseases (Eddy & Fogo, 2006). In the glomerulus, the effect of PAI-1 in mediating fibrosis is mostly by its ability to modulate extracellular matrix turnover (Eddy & Fogo, 2006). Thus, the mechanisms by which PAI-1 mediates fibrosis in the glomerulus versus interstitium are not identical, and they involve the interactions of parenchymal and infiltrating cells and cytokines, with variable net effects on ECM accumulation.

TGFβ is a key promoter of fibrosis and extracellular matrix synthesis. Transgenic animals overexpressing TGFβ developed progressive kidney injury. TGFβ induces the formation of Ang II and PAI-1 (Gaedeke, Peters, Noble, & Border, 2001). Animals transgenic for TGFβ developed progressive renal disease (Kopp et al., 1996). Animals which are genetically deficient for TGFβ develop lymphoproliferative disease, reflecting a loss of TGFβ immune regulatory effect (Christ et al., 1994). There is dose dependent effect of TGFβ: - at lower doses it promotes growth arrest and differentiation of podocytes whereas at higher doses it causes podocytes apoptosis which is mediated by Smad 7 signaling (Schiffer et al., 2001; Wu, Bitzer, Ju, Mundel, & Bottinger, 2005). Podocytes along with mesangial cells are important in maintaining the structure and function of the glomerulus. Loss of podocytes is a key factor in progressive kidney fibrosis.

PPARγ is a transcription factor and a member of the steroid superfamily which is involved in modifying numerous cytokines and growth factors including PAI-1 and TGFβ (Guan & Breyer, 2001). On activation, it binds to the retinoic acid X receptor, translocates to the nucleus and binds to proliferator activated response elements (PPREs) in selected target genes modifying their expression. Studies have shown the beneficial effect of PPARγ agonists in various animal models especially type 2 diabetes as they increase insulin sensitivity and lipid

metabolism thereby decreasing diabetic injury (Buckingham et al., 1998). In non-diabetic and non-hyperlipidemic animal models of CKD, PPARγ agonists have shown anti-fibrotic effects. PPARγ agonists reduce the development of sclerosis which is linked with a decrease in TGFβ and PAI-1 and also a decrease in the infiltrating macrophages, keeping the podocytes intact against injury (L. J. Ma, Marcantoni, Linton, Fazio, & Fogo, 2001; H. C. Yang, Ma, Ma, & Fogo, 2006).

D Podocyte Loss

Podocytes play an important role in maintaining glomerular structure and function. Together with the endothelial cells of the capillaries in the glomerulus and the glomerular basement membrane, they form a filtration barrier. While cooperating with mesangial cells, they support the structure and function of glomerulus. Podocytes do not proliferate. Loss of podocytes is one of the major factors resulting in sclerosis (Shankland, 2006). This principle was proven by conducting studies in experimental models of rats and mice where the podocyte specific injury was produced by the genetic manipulation of the podocyte to express a toxin receptor only on this cell (Matsusaka et al., 2005; Wharram et al., 2005). Injecting puromycin aminonucleoside causes podocyte loss; the severity of loss depends on the dose of the toxin. Animals eventually developed progressive sclerosis, but the available data from various studies demonstrated the effect of podocyte injury on endothelial and mesangial cells. It is possible that the injury can spread from injured podocytes to the intact podocytes within the glomerulus, which can initiate a vicious cycle leading to the progressive injury at glomerular level (Ichikawa, Ma, Motojima, & Matsusaka, 2005).

As mentioned earlier, podocytes do not proliferate and it is due to an increased expression of the cyclin dependent kinase inhibitor, p27kip1, which is a rate limiting step for the

growth response of the podocyte (Combs, Shankland, Setzer, Hudkins, & Alpers, 1998). Either too much or too little proliferation of the podocyte in response to genetic manipulation of p27kip1 is postulated to be detrimental. Recent studies of the molecular biology of the podocyte and the genes mutated in a different form of focal segmental glomerulosclerosis and nephrotic syndrome, such as nephrin, phospholipase C, α-actinin-4, and podocin have given a new area to study the mechanisms involved in progressive glomerulosclerosis. Nephrin is localized to the slit diaphragm of the podocyte and is tightly associated with CD2-associated protein (CD2AP) (Huber & Benzing, 2005). Nephrin is a protein that is important for the proper functioning of renal filtration barrier in the kidney. The renal filtration barrier consists of fenestrated endothelial cells, glomerular basement membrane and the podocytes of epithelial cells. Nephrin is present on the tip of the podocyte and maintains the normal relationship between the basement membrane and podocytes of the epithelial cells. CD2AP knockout mice develop congenital nephrotic syndrome. Mutation in α-actinin 4 leads to an autosomal dominant focal segmental glomerulosclerosis (FSGS) in adults (Kaplan et al., 2000). It is hypothesized that an altered actin-cytoskeleton interaction causes FSGS, which is through a gain-of-function mechanism in contrast to a loss-of-function mechanism in diseases caused by the mutation in nephrin. Podocin, another gene associated with podocytes, when mutated results in autosomal recessive FSGS with childhood onset and finally progressing to end stage renal disease (Boute et al., 2000). Studies have shown that in various experimental models, there is acquired disruption or polymorphism of these complex interacting molecules. In puromycin aminonucleoside nephropathy, a model of FSGS, there is alteration of nephrin localization and organization (Kawachi et al., 2000). Morel et al. have shown the decreased glomerular nephrin expression in Dahl salt sensitive rats on high salt diet (Worou et al., 2015).

E Dyslipidemia

People with obesity and chronic kidney disease have dyslipidemia and a greater risk for cardiovascular events (Cases & Coll, 2005). In rats, abnormal lipid profile is important in modulating glomerular sclerosis. In experimental models where excess cholesterol was added, glomerular injury was increased (Keane, Mulcahy, Kasiske, Kim, & O'Donnell, 1991). Recent clinical trials support the notion that abnormal lipids are associated with an increased loss of GFR, and treatment with statins not only improves cardiovascular events but also helps in ameliorating chronic kidney disease events (Tonelli, Moye, Sacks, Cole, & Curhan, 2003).

F Proteinuria

Proteinuria is a significant marker for renal damage and cardiovascular morbidity and mortality. Nephrin is a key protein which is involved in the maintenance of the glomerular filtration barrier in the kidney. Reduced expression of nephrin leads to a leaking of albumin in urine which is a marker for renal damage. Mutations in nephrin are associated with a congenital nephrotic disorder in infants that is characterized by massive proteinuria. Proteinuria contributes to progressive renal inflammation and is often associated with a worst prognosis (Shankland, 2006). In the proteinuric state, the other components, which are filtered in the urine such as oxidized proteins, cause more injury to tubular epithelial cells and activate pro-inflammatory cytokines and chemokines (Perico, Codreanu, Schieppati, & Remuzzi, 2005). Complement and various lipoproteins are also present in the urine in the proteinuric state and can cause reactive oxygen species activation. Proteinuria may activate various profibrotic pathways due to its ability to increase NF-κB and also other pathways (Abbate, Zoja, & Remuzzi, 2006).

Drugs like ACEis and ARBs have been proven to ameliorate the progression of end organ injury. It has not been proven yet whether the beneficial effects of these drugs are due to a

reduction in proteinuria as they have multiple parallel effects which aim to reduce fibrosis (Abbate et al., 2006).

Renal Hemodynamics in Obesity

Clinically as well as in the experimental models, obesity increases the risk of renal damage (D'Agati et al., 2016; Kovesdy et al., 2017). Obesity is associated with abnormal renal hemodynamics in the form of increased glomerular capillary pressure. Increased glomerular capillary pressure can increase the propensity to cause renal damage due to barotrauma and stretch to the glomerular linings (Bondar et al., 2011; Riser et al., 1992). Understanding the regulation of the renal microcirculation is important to understand glomerular and renal injury.

Normally, the resistance of both afferent and efferent arterioles regulates glomerular capillary pressure. Afferent arteriolar resistance is regulated by mechanisms similar to those regulating other arterioles including plasma Ang II, sympathetic nervous system, and myogenic response (Navar, 2014). In addition, Af-Arts are also regulated by two autoregulatory intrinsic feedback mechanisms: 1) tubuloglomerular feedback (TGF) that causes Af-Art constriction in response to an increase in NaCl in the macula densa, via the sodium–potassium-2-chloride cotransporter-2 (NKCC2) and 2) connecting tubule glomerular feedback (CTGF) mechanism that causes Af-Art dilation in response to an increase in NaCl in the CNT segment of the nephron via epithelial sodium channel (ENaC) (Ren, D'Ambrosio, Garvin, Wang, & Carretero, 2009; Ren, Garvin, Liu, & Carretero, 2009). The CNT segment of the nephron comes in contact with the afferent arteriole and this forms the basis for tubule to arteriole signaling resulting in change in the afferent arteriolar diameter. During in-vitro perfusion of microdissected Af-Art and the adherent CNT, increasing the NaCl concentration in the perfusate of the CNT significantly dilated the preconstricted Af-Art and this phenomenon is known as CTGF (Ren, D'Ambrosio, et

al., 2009). The phenomenon of CTGF was abolished upon blocking the epithelial sodium channel specifically with benzamil confirming that epithelial sodium channels mediate CTGF. As far as the mechanism of CTGF is concerned, it is known that CTGF is initiated by epithelial sodium channels present at the CT segment of nephron and is mediated by arachidonic acid metabolites. Increasing the sodium concentration in the CNT stimulates the release of prostaglandins and epoxyeicosatrienoic acids (Ren, D'Ambrosio, et al., 2009). Released prostaglandin (PG) E2 from the CT binds to the prostaglandin E2 receptor 4 (EP4) on the afferent arteriole wall and induces its dilation. CTGF is increased during high NaCl intake in Dahl SS rats and by hormones that stimulate the ENaC, such as aldosterone and Ang II (H. Wang et al., 2017). Also in unilateral nephrectomy, CTGF was increased in the remnant kidney (Monu et al., 2017). Since CTGF is an afferent arteriolar dilator and TGF is an afferent arteriolar constrictor, it is possible that they can mutually influence each other. The interaction between TGF and CTGF is evident from studies showing that by blocking the CTGF pharmacologically with benzamil, TGF gets further potentiated which further suggests that CTGF indeed opposes the TGF mechanism, and it is more evident in the model of salt sensitive rats and unilaterally nephrectomized rats.

In obesity, there seems to be an alteration in the normal renal hemodynamics as there is increased glomerular capillary pressure and increased renal blood flow in obese animals as well as obese human beings (Bondar et al., 2011; Leggio et al., 2017). Because of this alteration in renal hemodynamics, it is possible that feedback mechanisms such as TGF and CTGF might be playing a role in causing this. There are several studies pointing out the possibility of TGF attenuation in obesity but none of this has been confirmed yet.

Zucker Obese Rats

Zucker obese (ZO) rats are the best known and the most widely used animal model for genetic obesity. The *fa* mutation was discovered in 1961 by Lois Zucker in a cross between Merck M-strain and Sherman rats (Zucker & Zucker, 1963). The animals that are homozygous for the *fa* allele, the *fa/fa* Zucker rats or ZO rats become obese at 3-5 weeks of age. These animals have a mutation in the leptin receptor that is responsible for their characteristic phenotype. Leptin is a hormone which is produced by adipose tissue and released in the circulatory system and is involved in maintaining the energy balance. ZO rats are morbidly obese and hyperphagic. Hyperphagia is mostly seen in the growth period of animals, i.e. during the first 16 weeks of age. ZO rats develop proteinuria at 12 weeks of age and by 14 weeks of age, the body composition of these rats become 40% weight lipid. These animals are insulin resistant, normoglycemic and hyperinsulinemic (Kasiske et al., 1992). There are conflicting results whether these animals are hypertensive compared to their lean littermates. Various studies have shown that upon high salt diet treatment these animals become hypertensive indicating that they develop salt sensitive hypertension and hence they are used as a rodent model to study obesity related kidney dysfunction and hypertension.

We used ZO rats to study obesity related kidney damage in our present study. This model exhibits many phenotypic traits common for obesity related kidney damage observed in human population and is associated with a progressive decline of renal function and albuminuria. These rats exhibit hyperinsulinemia but are normoglycemic representing the prediabetic state in the human beings (Kasiske et al., 1992). Zucker lean (ZL) rats were used as the control animals in our experiments.

Based on all the findings mentioned above, we hypothesized that in ZO rats on high salt diet Ac-SDKP prevents renal damage by decreasing renal inflammation, fibrosis, and glomerulosclerosis as well as delays the onset of hypertension.

CHAPTER 3

RENAL PROTECTIVE EFFECTS OF N-ACETYL-SERYL-ASPARTYL-LYSYL-PROLINE (Ac-SDKP) IN OBESE RATS ON A HIGH-SALT DIET

This manuscript corresponds to Aim 1 and 2 and is focused on the hypothesis that Zucker obese rats on a high salt diet develop renal damage, inflammation, and fibrosis that are prevented by Ac-SDKP treatment.

A Manuscript published in the *American Journal of Hypertension*

Maheshwari M, Romero CA, Monu SR, Kumar N, Liao TD, Peterson Ed, and Carretero OA (2018).

"Renal Protective Effects of N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) in Obese Rats on a High-Salt Diet." Am J Hypertens, 2018 [Epub ahead of print]

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Abstract

Obesity is a public health problem associated with salt sensitive hypertension, kidney inflammation, and fibrosis. N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) is a tetra peptide with anti-inflammatory and anti-fibrotic properties; however, its effect on preventing kidney damage in obesity is unknown. We hypothesized that Zucker obese (ZO) rats on a high-salt (HS) diet develop renal damage, inflammation, and fibrosis and this is prevented with Ac-SDKP treatment. Zucker Lean (ZL) and ZO rats (8 weeks old) were treated with Ac-SDKP (1.6 mg/kg/day) while maintained on either a normal-salt (NS; 0.4%) or HS (4%) diet for 8 weeks. Systolic blood pressure (SBP), albuminuria, renal inflammation, and fibrosis were evaluated. HS diet increased macrophage infiltration in the kidneys of both ZL and ZO rats but was significantly higher in ZO rats receiving the HS diet (ZL+NS, 13.9±1.3 *vs* ZL+HS, 19.14±1.5 and ZO+NS, 25.5±1.4 *vs* ZO+HS, 87.8 ± 10.8 cells/mm^2*; P <0.05*). Ac-SDKP prevented macrophage infiltration in ZO rats (ZO+HS+Ac-SDKP, 32.18±2.4 cells/mm$^{2;}$ *P* <0.05). Similarly, glomerulosclerosis, cortical and medullary interstitial fibrosis were increased in ZO rats fed the HS diet, and Ac-SDKP attenuated these alterations (*P* <0.05). SBP was increased in ZO rats fed the HS diet (ZO+NS, 121.3±8.9 *vs* ZO+HS, 164±6.9 mmHg; *P <0.05*), and significantly decreased with Ac-SDKP treatment (*P* =0.004). Albuminuria was higher in ZO rats than in ZL rats; however, neither HS nor Ac-SDKP treatment affected it. We concluded that Ac-SDKP treatment in ZO rats fed a HS diet prevented renal damage by reducing inflammation, fibrosis, and BP.

Background

Obesity is a public health problem in the United States, almost 70% of the population is overweight; among them, approximately 35% are obese, with a body mass index above 30 kg/m^2 (J. E. Hall et al., 2015). Obesity is an important risk factor for end-stage renal disease due to its strong association with diabetes and hypertension. The incidence of kidney damage associated to obesity has increased 10-fold in the last 15 years and is expected to rise further in the coming years (Kovesdy et al., 2017; Mathew et al., 2011). Obesity is also linked to salt-sensitive hypertension in both humans and animals (Ali et al., 2015; DeMarco et al., 2014). In the obese population, salt-sensitive hypertension is strongly associated with the progression of target-organ damage, including end-stage renal disease (Quigley et al., 2009). The underlying mechanism of obesity-related salt sensitivity and its association with renal injury remains unclear. However, inflammation plays a key role in the development of hypertension and kidney damage associated with obesity (Harrison et al., 2011; Schiffrin, 2014). Previous studies have shown that, in obesity, renal injury was associated with glomerulosclerosis, tubule-interstitial damage, inflammation and albuminuria. High-salt (HS) intake further aggravated these renal changes (M. E. Hall et al., 2014). N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) is a tetra-peptide, naturally present in many tissues including kidney (Junot et al., 1999), that is released from its precursor thymosin β4 by 2 enzymatic steps that are mediated by meprin-α and prolyl oligopeptidase enzymes (Cavasin et al., 2004; Kumar et al., 2016). Ac-SDKP is hydrolyzed mainly by angiotensin-converting enzyme (ACE), and its concentration in plasma, urine, kidney, and heart is increased by ACE inhibitors (ACEi) (Azizi et al., 1996). We previously demonstrated that some of the anti-inflammatory and anti-fibrotic effects of ACEi are mediated by an increase in endogenous Ac-SDKP concentration (Peng et al., 2007). Studies using several experimental

animal models have demonstrated that Ac-SDKP has anti-inflammatory and anti-fibrotic properties (Rhaleb et al., 2011; Worou et al., 2015) and that a decrease in endogenous Ac-SDKP levels promoted fibrosis in the kidney and heart (Cavasin et al., 2007). Recently, we have also shown that Ac-SDKP can delay the onset of hypertension in systemic lupus erythematosus (Nakagawa et al., 2017). However, the effect of Ac-SDKP on obesity-related kidney damage and hypertension is still unknown.

Zucker Obese (ZO) rats exhibit many phenotypic traits that are common in the obesity observed in humans such as hyperinsulinemia with normoglycemia, and is associated with albuminuria and a progressive decline of renal function (Kasiske et al., 1992).

Therefore, we hypothesized that Zucker obese (ZO) rats on a HS diet develop renal damage, inflammation, and fibrosis which are prevented with Ac-SDKP treatment.

Materials and Methods

Animals

Male ZL and ZO rats at 5 weeks of age (Charles River Laboratories, Wilmington, MA, USA) were housed in an air-conditioned room with a 12-hour light/dark cycle and received standard laboratory rat chow and tap water. Rats were allowed 7 days to acclimatize to the new environment before the experiments were performed. All surgical procedures were performed under anesthesia (50 mg/kg of sodium pentobarbital, intraperitoneal). The study was approved by the Henry Ford Hospital Institutional Animal Care and Use Committee.

Experimental Protocols

ZL and ZO rats (8 weeks old) were placed on either a normal-salt (NS; 0.4% NaCl) or HS (4% NaCl) diet (Teklad diets, Harlan, Madison, WI) and were subcutaneously infused with vehicle (0.01 N acetic acid 0.9% saline solution) or Ac-SDKP (1.6 $mg \cdot kg^{-1} \cdot day^{-1}$) for 8 weeks

using osmotic mini-pumps (Alzet, Cupertino CA). ZL and ZO rats were divided into 6 groups: 1) NS infused with vehicle (NS+vehicle, n = 6); 2) HS infused with vehicle (HS+vehicle, n = 6); and 3) HS infused with Ac-SDKP (HS+Ac-SDKP, n=6). Blood pressure was measured weekly with a tail-cuff method; 24-hour urine collection was carried out for urinary Ac-SDKP, albumin and sodium excretion. At the end of the experiment, the animals were sacrificed, and tissues were weighed and collected for biochemical and histological studies.

Systolic Blood Pressure

SBP was measured in conscious rats with a noninvasive computerized tail-cuff system (CODA, Kent Scientific, Torrington, CT), as described previously (Liao et al., 2010).

Urinary Ac-SDKP, Sodium Excretion and Albuminuria

Animals were placed in metabolic cages for a 24-hour period for acclimatization before 24 hours urine collection. The ACEi captopril was applied to the collecting funnels and tubes at the final concentration of 10^{-5} M to prevent Ac-SDKP degradation by urinary ACE. The total volume of collected urine was measured; aliquots were prepared and centrifuged twice at 4°C and 1200 g for 10 minutes (Eppendorf centrifuge 5415R). The supernatants were filtered and stored at -80°C until further analysis. Urinary Ac-SDKP was measured using competitive enzyme linked immunosorbent assay (ELISA) kit according to the manufacturer's protocol (SPI Biolaboratories, France) as previously described (Liao et al., 2008). Urinary albumin was determined with an ELISA kit according to the manufacturer's protocol (GenWay Biotech Inc, San Diego). The 24-hour sodium excretion values were calculated from the 24-hr urine volumes and the sodium concentrations measured with a Nova Biomedical 1 electrolyte analyzer (Waltham, MA). Urine albumin excretion was calculated as the urine albumin concentration multiplied by the 24-hour urine volume output.

Renal Macrophage Infiltration

Frozen kidney sections (6 μm) were fixed with acetone (4°C) for 20 minutes. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide. Nonspecific antibody binding was blocked with 1% bovine serum albumin. Primary antibody mouse anti-rat CD68, a marker for macrophages (clone: ED-1, 1:200, Millipore), was applied, and samples were incubated overnight at 4°C. The following day, sections were incubated with biotinylated secondary horse anti-mouse immunoglobulin G antibodies. Immunoreactivity was detected with ABC peroxidase kit (Vectastain Elite, Vector Laboratories, Burlingame, CA) and visualized with 3-amino-9-ethylcarbazole (Zymed Laboratories, San Francisco, CA). Reddish-brown staining was considered positive. Sections were counterstained with hematoxylin to see the nucleus of the cell. Twenty randomly chosen regions of the section were examined under the ×20 objective of a Nikon Eclipse E600 microscope and evaluated with a computerized image analysis system (Microsuite Biological Imaging, Olympus America, Center Valley, PA). Positive cells with clearly visible nuclei were counted at high power for each section and expressed as cells per square millimeter. All histological studies were performed with blinded analysis.

Renal Fibrosis

Picrosirius Red staining (PSR) was used to quantify the renal cortical and medullary interstitial collagen deposition as described previously (Nakagawa et al., 2012). Sequential 4-μm paraffin-embedded sections were stained. Briefly, tissues were postfixed in Bouin's fluid and then stained with 0.1% Picrosirius Red for 1 hour. Samples were then washed twice in 0.5% acetic acid. Nuclei were counterstained with hematoxylin. For cortical and medullary renal interstitial collagen fraction, 30 images were taken with the ×20 objective of a Nikon Eclipse E600 microscope with Nikon DS-Ri1 digital camera (Nikon Instruments Inc.). Images were

analyzed by computerized image analysis (Microsuite Biological imaging software, Olympus America) and expressed as the ratio of the area stained positive for collagen to the entire area of the captured field.

We also determined the total collagen content of the renal cortex using the hydroxyproline assay, as described previously (Peng et al., 2007). Briefly, samples were dried and weighed, homogenized, and hydrolyzed with 6 N HCI for 16 hours at 110°C. A standard curve of 0 to 5 μg of hydroxyproline was used. Data were expressed as micrograms of collagen per milligram of dry weight, assuming that collagen contains an average of 13.5% hydroxyproline.

Glomerular Injury

The glomerular matrix was evaluated by periodic acid-Schiff (PAS) staining (Sigma), according to the manufacturer's protocol. A trans-mural section was taken from the upper mid-kidney section. Sequential 4-μm paraffin-embedded sections were stained with PAS. Glomeruli (30 to 50) within the randomly chosen fields of the renal cortex were photographed under the ×20 objective. The dark purple color within the glomeruli was considered a positive signal representing the extracellular matrix. The degree of glomerulosclerosis was determined as a percentage of the glomerular tuft area.

Intraperitoneal Glucose Tolerance Test (ipGTT)

On week 8 of the treatment, rats were fasted overnight, blood samples were taken from the tail vein, and glucose was measured using a glucometer (Bayer Contour Blood glucose meter) at 0 (fasting), and 15, 30, 60, 90 and 120 minutes after giving an intraperitoneal injection of glucose (2 g/kg). The total area under the curve (AUC) for glucose during the ipGTT (2-hr glucose area under curve) was calculated using the Graph pad Prism software version 5.01.

Statistical Analysis

A nonparametric two-sample Wilcoxon test was used to compare contrasts of interest in all the data. To adjust for multiple testing, Hochberg's method was used to determine the significance. The adjustment was made on groups of similar tests. A *P*-value less than 0.05 was considered evidence of significant differences.

Results

Body Weight and Urinary Ac-SDKP

ZO rats showed a significantly higher body weight than ZL rats (Table 2). Neither HS diet nor Ac-SDKP treatment showed any effect on body weight in ZL or ZO rats. Compared to ZL rats, ZO rats showed significant glucose intolerance (Table 2). We observed that the high-salt diet further increased the glucose intolerance in ZO rats, but it had no effect on ZL rats. Ac-SDKP treatment showed no effect on glucose intolerance. As we expected, 24-hr urinary Ac-SDKP excretion was significantly higher (10 to 20-fold) in ZL and ZO rats receiving Ac-SDKP treatment than in the vehicle-treated groups. No effects of high salt were observed in Ac-SDKP excretion. Upon high salt diet, ZO rats have significantly higher urine volume compared to ZL rats but Ac-SDKP treatment did not affect it.

Table 2 Effect of High Salt and Ac-SDKP in Zucker Rats at 8 weeks of HS Diet and Ac-SDKP Treatment

	Zucker Lean Rats			Zucker Obese Rats		
Parameters	Normal Salt	High Salt	High Salt+Ac-SDKP	Normal Salt	High Salt	High salt+Ac-SDKP
Body Weight (grams)	357.8±12.5	354.67±10.2	351.5±10.46	637.4±14.9$^{\neq}$	587.3±17.1	576±16.64
Renal Collagen Content (µg/mg dry weight)	17.7±0.65	25.2±2.6§	17.1±1.14¶	20.4±1.2	33.87±6.19*	21.46±2.5†
Albuminuria (mg/24hr)	0.53±0.23	0.97±0.43	2.25±1.34	62.3±22.97$^{\neq}$	85.73±15.1	43.47±15.65
Urinary Ac-SDKP (nmol/24hr)	1.35±0.06	1.73±0.21	73.56±7.3$^{\pm,\P}$	3.56±1.38	2.6±0.37	117.63±23.75$^{\#,\dagger}$
Urinary Sodium (mMol)	1.76±0.08	8.14±1.3§	7.2±0.6$^{\pm}$	4.1±1.2	13.5±0.8*	16.4±1.5$^{\#}$
24 hr Urine Excretion (ml)	12.3±1.43	19.2±1.9§	18.3±1.8	24.3±8.6	49.8±4.6*	47±5.8
Fasting Glucose (mg/dl)	74.2±3.3	85.8±3.7	81.6±4.6	94.8±2.5	96.6±4.5	98.3±6.0
GTT (Area Under Curve)	1516±125.6	1349±74.36	1276.75±111.6	2188.5±126.5$^{\neq}$	2537.8±71.9*	2383.67±78.9

*P<0.05 (n=6) ZO+NS *vs* ZO+HS; †P<0.05 ZO+HS *vs* ZO+HS+Ac-SDKP ; $^{\neq}$P<0.05 ZL+NS *vs* ZO+NS; § P<0.05 ZL+NS *vs* ZL+HS; $^{\pm}$P<0.05 ZL+NS *vs* ZL+HS+Ac-SDKP;¶ P<0.05 ZL+HS *vs* ZL+HS+Ac-SDKP; $^{\#}$ P<0.05 ZO+NS *vs* ZO+HS+Ac-SDKP
GTT: Glucose Tolerance Test

Renal Inflammation

Macrophage infiltration was examined by immunohistochemistry. Compared to ZL rats, ZO rats showed increased numbers of CD68+ positive cells (macrophages) infiltrating the renal parenchyma (Figure 4). The HS diet significantly increased the number of infiltrating macrophages in both ZL and ZO rats, but this increase was markedly higher in ZO rats fed HS. Ac-SDKP treatment significantly decreased the infiltrating renal macrophages in ZO rats but not in ZL rats. These data indicated that HS diet exaggerated the renal inflammation, markedly in obese animals, and that was prevented by Ac-SDKP treatment.

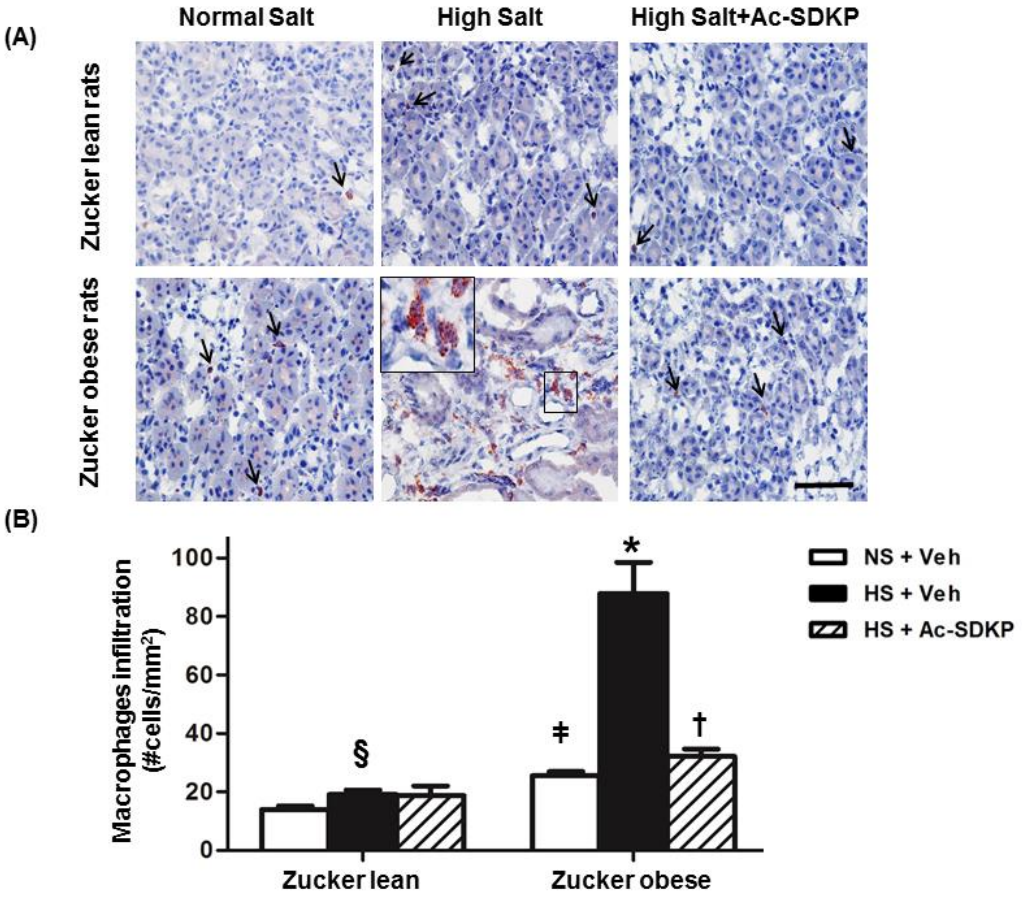

Figure 4. Effect of Ac-SDKP on Renal Macrophages Induced by a HS Diet in Obesity. (A) Representative images of renal macrophages infiltration in ZL and ZO rats fed a HS diet and receiving Ac-SDKP (Scale bar = 50 μm). Red staining in the cytoplasm indicates a positive immunohistochemistry staining for macrophages (anti-CD68 antibody). A HS diet increased the CD68+ positive cells in the kidney, and this increase was markedly important in obese rats. Ac-SDKP prevented macrophages infiltration in obese rats. Inset is showing a group of macrophages at higher magnification in the interstitial renal space. (**B**) Quantitative analysis of macrophages infiltration. Both in ZO and ZL rats, the HS diet increased macrophages infiltration in the kidney. Ac-SDKP significantly reduced HS-induced renal macrophage infiltration in ZO rats but not in ZL rats. Data were calculated as the number of cells per millimeter^2 and expressed as the mean ± standard error of measurement. N = 6 in each group. $^{\S}P < 0.05$ ZL + NS *vs* ZL + HS, $^{ǂ}P < 0.05$ ZL + NS *vs* ZO + NS, $^{*}P < 0.05$ ZO + NS *vs* ZO + HS, $^{\dagger}P < 0.05$ ZO + HS *vs* ZO + HS + Ac-SDKP. Abbreviations: Ac-SDKP, N-acetyl-seryl-aspartyl-lysyl-proline; HS, high-salt; ZL, Zucker lean; ZO, Zucker obese.

Renal Fibrosis

Both the cortical and medullary interstitial fibrosis quantified by PSR staining was similar in ZL and ZO rats fed a normal diet at 16 weeks of age (Figure 5). A high-salt diet in ZO

rats showed a significant increase in the cortical and medullary interstitial fibrosis compared to ZL rats and Ac-SDKP treatment attenuated this increase in ZO rats. In addition, analysis of total renal collagen content by a hydroxyproline assay confirmed our finding that a high-salt diet significantly increased the total renal collagen content in both ZL and ZO rats, which was significantly decreased by Ac-SDKP treatment (Table 2).

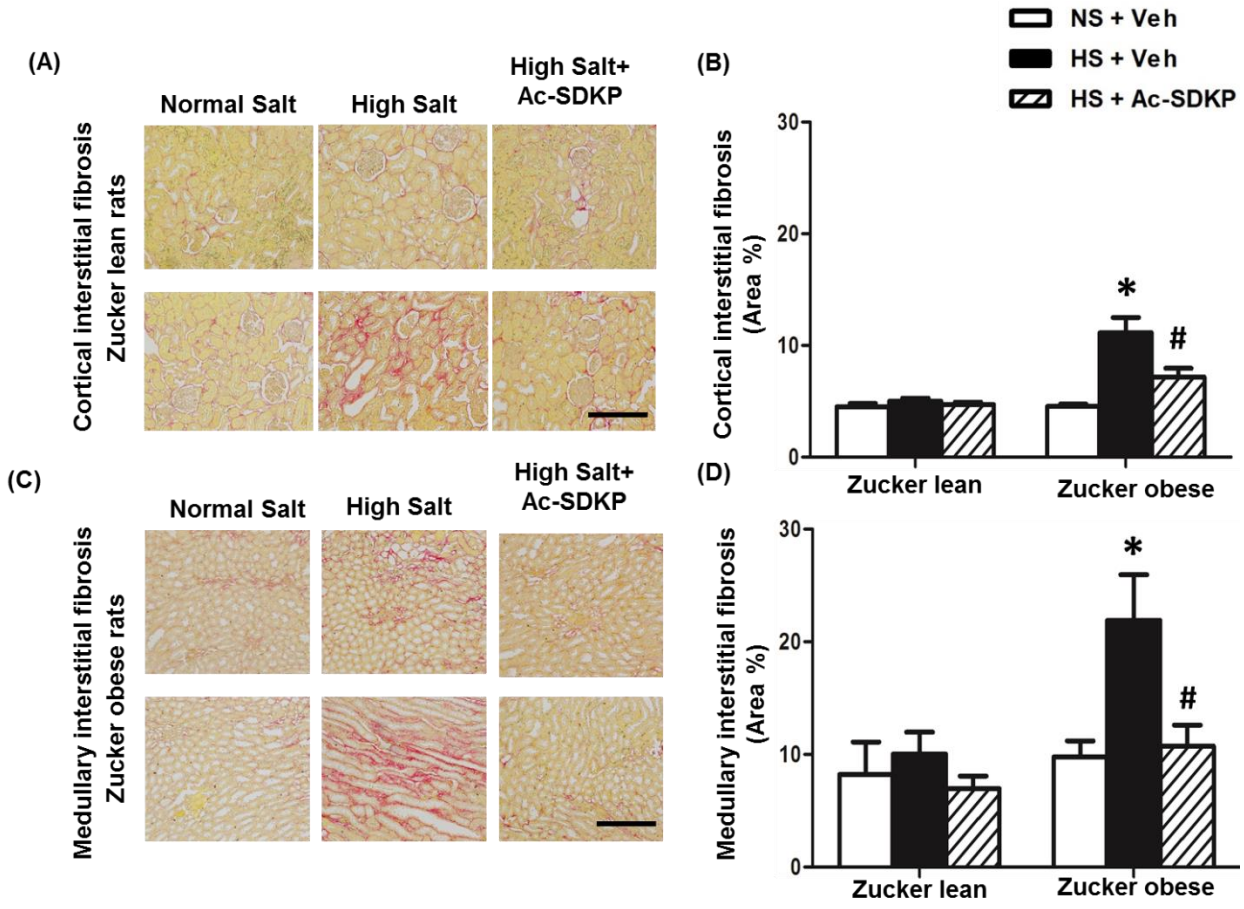

Figure 5. Effect of Ac-SDKP on Renal Cortical and Medullary Interstitial Fibrosis in Obese Rats Fed a HS Diet. (A and C) Representative images of renal cortical and medullary interstitial fibrosis. Red color indicates collagen deposition revealed by Picrosirius Red Staining. (Scale bar = 100 μm). Interstitial fibrosis was increased in ZO rats fed with HS in both the cortex and medulla, and that was prevented by Ac-SDKP. **(B and D)** Quantitative data analysis. In ZO rats, Ac-SDKP significantly prevented HS-induced renal cortical and medullary collagen deposition. Data are calculated as a percentage of the fibrotic area and expressed as the mean ± SEM. N=6 in each group. *P<0.05 ZO+NS *vs* ZO+HS, $^{\#}$ P<0.05 ZO+HS *vs* ZO+HS+Ac-SDKP.

Glomerular Damage

The effect of Ac-SDKP on glomerulosclerosis was assessed by Periodic Acid-Schiff staining (PAS). Compared to ZL rats, ZO rats exhibited glomerulosclerosis, which was detected as dark purple regions of extracellular matrix deposition within the glomerular tufts (Figure 6). A high-salt diet for 8 weeks showed a significant increase in glomerulosclerosis in ZO rats but not in ZL rats, and this increase was significantly attenuated by treatment with Ac-SDKP in ZO rats.

Albuminuria, which is a marker of glomerular damage, was significantly higher in ZO rats compared to ZL rats (Table 2). Interestingly, there was a trend for HS to increase albuminuria in ZO rats and Ac-SDKP treatment decreased it but it did not reach the statistical significance.

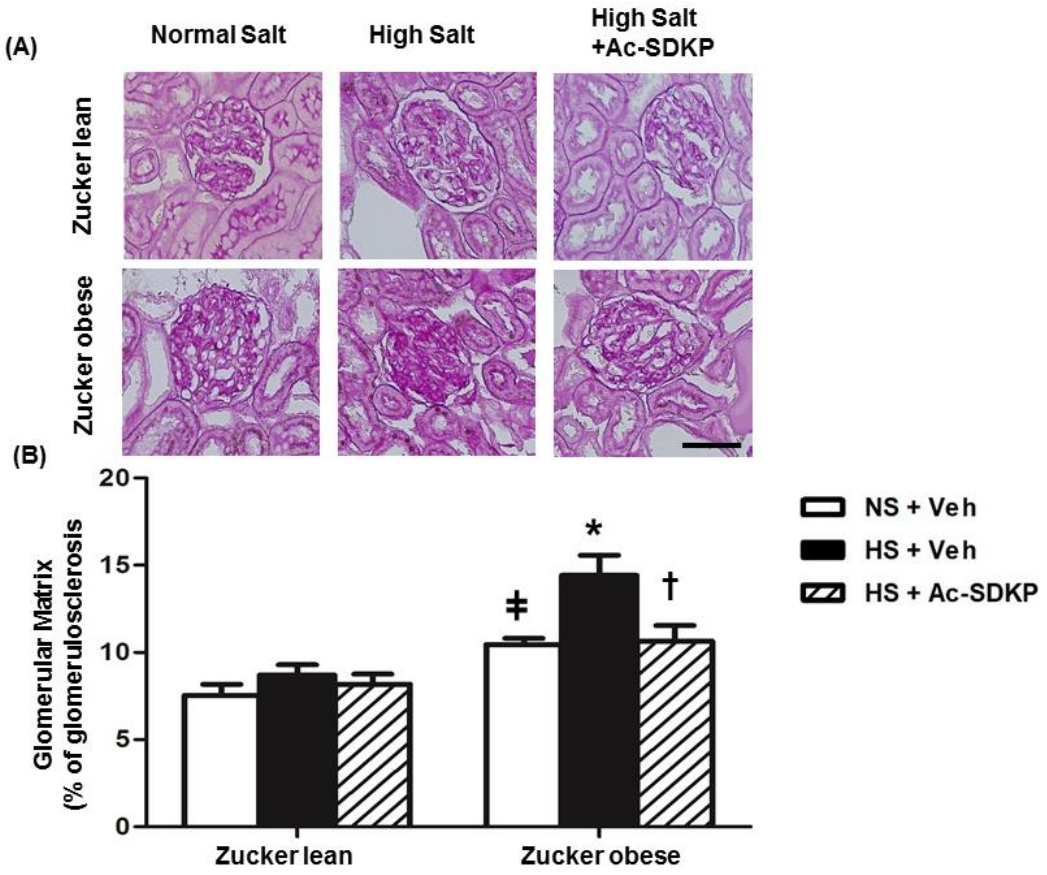

Figure 6. **Effect of Ac-SDKP on Glomerular Matrix Deposition in Obese Rats Fed a HS**

Diet. (A) Representative images of the glomerular matrix. Dark-purple regions indicate the extracellular matrix stained within the glomerular tufts by Periodic Acid-Schiff staining. Shown are images captured using the x20 microscope objective. Scale bar = 25 μm. Glomerulosclerosis was increased in ZO rats in comparison with the ZL control. A HS diet increased the glomerulosclerosis in ZO rats, and that was prevented by Ac-SDKP. **(B)** Quantitative data analysis. In ZO but not in ZL rats, glomerulosclerosis was significantly increased by a HS diet compared to a NS diet. Ac-SDKP significantly prevented HS-induced glomerulosclerosis in ZO rats. Data are expressed as the mean ± standard error of measurement. N = 6 in each group. $^{\ddagger}P < 0.05$ ZL + NS *vs* ZO + NS, $^{*}P < 0.05$ ZO + NS *vs* ZO + HS, $^{\dagger}P < 0.05$ ZO + HS *vs* ZO + HS + Ac-SDKP. Abbreviations: Ac-SDKP, N-acetyl-seryl-aspartyl-lysyl-proline; HS, high-salt; NS, normal-salt; ZL, Zucker lean; ZO, Zucker obese.

Systolic Blood Pressure (SBP)

At baseline conditions, no difference was found in the systolic blood pressures (SBP) of the ZL and ZO rats (Figure 7). ZO rats started showing a significant increase in SBP from week 2 of the HS diet (10 weeks of age), and it continued to increase until week 8 (16 weeks of age). In contrast, ZL rats did not show any increase in SBP with the HS diet intake. Ac-SDKP treatment did attenuate the increased blood pressure in ZO rats fed a high-salt diet, but it did not show any effect on blood pressure in ZL rats. In the last week of the protocol, Ac-SDKP still decreased the blood pressure in ZO rats, but the difference with rats receiving HS was not statistically significant.

The quantification of sodium excretion shows that a high-salt diet increases sodium excretion in both ZL and ZO rats and that Ac-SDKP treatment did not affect it, indicating that there is no difference in salt intake in these groups of animals upon Ac-SDKP treatment (Table 2).

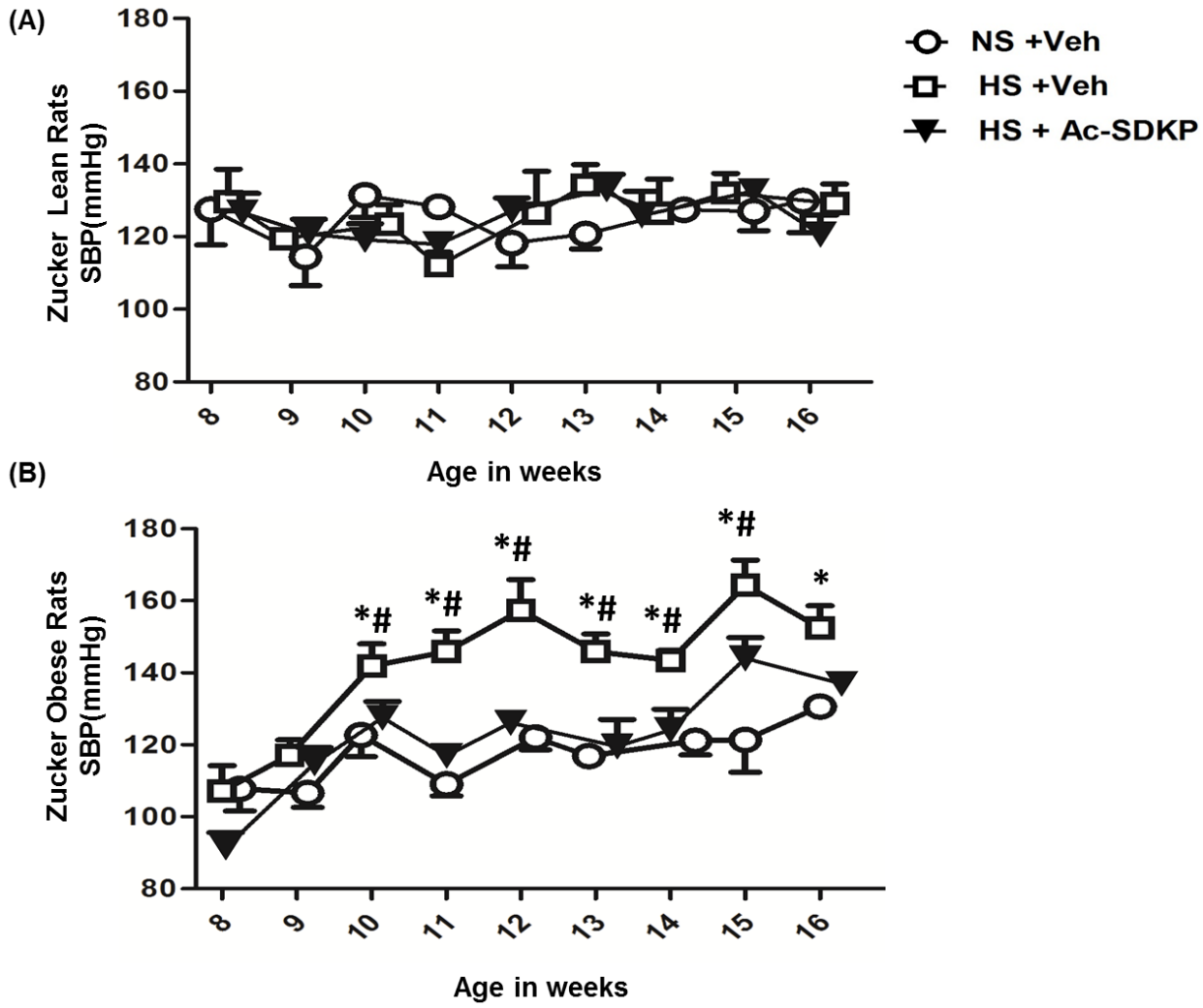

Figure 7. Effect of Ac-SDKP on Systolic Blood Pressure (SBP) in Obese Rats Fed a HS Diet. SBP was measured weekly in conscious rat with a tail cuff method. In ZO rats but not in ZL rats, the HS diet increased significantly SBP compared to the NS diet. Ac-SDKP significantly decreased the HS- induced high blood pressure in ZO rats. Data are expressed as the mean ± SEM. N=6 in each group. *P<0.05 ZO+NS *vs* ZO+HS, # P<0.05 ZO+HS *vs* ZO+HS+Ac-SDKP.

Discussion

In the current study, we examined the protective effects of Ac-SDKP on HS-induced kidney damage in obesity. Our results showed that HS diet aggravates renal damage in ZO rats, inducing renal macrophage infiltration, interstitial fibrosis, and glomerulosclerosis along with hypertension and that Ac-SDKP prevented all these effects. Additionally, Ac-SDKP reduced both renal cortical and medullary fibrosis but failed to have any beneficial effect on albuminuria.

Obese individuals are predisposed to develop salt sensitive hypertension and renal damage. In obesity, the kidneys initially become inflamed and eventually develop fibrosis; this effect is further aggravated with HS intake. Ac-SDKP is a naturally occurring tetra-peptide that has anti-inflammatory and anti-fibrotic properties in several models of cardiovascular and renal diseases (Worou et al., 2015; F. Yang et al., 2004) but its effect on obesity-related kidney damage is currently unknown. Many studies have provided evidence that infiltrating macrophages play a vital role in mediating obesity related kidney damage (Coimbra et al., 2000; Tang, Yan, & Zhuang, 2012). Our data indicated macrophage infiltration was markedly increased in the kidney of ZO rats compared to the ZL rats at 16 weeks of age, similar to previous studies (Lavaud et al., 1996; Rodriguez-Iturbe, Quiroz, Shahkarami, Li, & Vaziri, 2005). HS diet further exaggerated macrophage infiltration in both ZL and ZO rats. Findings of HS induced macrophage infiltration is in line with other studies, wherein HS intake induced macrophage infiltration (Wei et al., 2017; Worou et al., 2015). Macrophage infiltration leads to the release of proinflammatory cytokines and chemokines such as tumor necrosis factor α, IL-6, IL-1β, monocyte chemoattractant protein-1 (MCP-1) (Arango Duque & Descoteaux, 2014). We report here that Ac-SDKP treatment significantly reduced macrophage infiltration in ZL and ZO rats fed on HS. The beneficial effect exerted by Ac-SDKP on the reduction of macrophage infiltration in ZO rats is similar to our previously reported study, wherein Ac-SDKP prevented macrophage infiltration in both the Dahl salt-sensitive and resistant rats fed a HS diet (Worou et al., 2015).

Generally, renal fibrosis is the end result of inflammation, and the same is evident in our current study. Renal fibrosis (total renal collagen content) was increased in both ZL and ZO rats fed a HS diet, and Ac-SDKP treatment prevented this increase. Several mechanisms may be

mediating the anti-fibrotic effects of Ac-SDKP. Along with the anti-inflammatory effects, it is known that Ac-SDKP decreases transforming growth factor-beta/Smad signaling, which could be the underlying mechanisms associated with the decreased fibrosis (Lavaud et al., 1996; F. Yang et al., 2004). Interestingly, we also noticed increased total renal collagen content in HS-fed ZL rats, indicating high salt, independent of obesity and hypertension per se, can exert mild renal damage. In ZO rats, increases in glomerulosclerosis are attributed to the high glomerular capillary pressure followed by the infiltration of immune cells. In our study, glomerulosclerosis was also significantly increased in the ZO rats, and it was further aggravated by a HS diet. Since Ac-SDKP has been shown to reduce glomerulosclerosis in numerous studies, including db/db mice and Dahl salt-sensitive rats (Shibuya et al., 2005; Worou et al., 2015), we investigated whether Ac-SDKP reduced glomerulosclerosis in ZO rats and found that indeed Ac-SDKP treatment significantly reduced glomerulosclerosis in ZO rats.

Parallel to glomerulosclerosis, ZO rats also develop albuminuria, but the HS diet did not worsen it in either ZO rats or lean controls. Although Ac-SDKP treatment decreased glomerulosclerosis, it failed to ameliorate albuminuria in the ZO rats. Ac-SDKP treatment has been shown to decrease albuminuria in several models of renal diseases, such as Dahl salt-sensitive rats, 5/6 nephrectomy, and deoxycorticosterone acetate-salt induced hypertension (Liao et al., 2010; Rhaleb et al., 2011; Worou et al., 2015). One of the possible explanations for Ac-SDKP not showing any beneficial effect on albuminuria could be related to the animal model itself, as ZO rats are obese, hyperinsulinemic, and glucose-intolerant. Thus, the mechanism of albuminuria in this animal model could be different from that observed in previously reported models. Recent findings have suggested that urinary albumin excretion could result either because of the defect in the glomerular filtration barrier and/or defect in the albumin absorption

in the proximal tubule (Dickson, Wagner, Sandoval, & Molitoris, 2014; Vallon, 2011). Since Ac-SDKP treatment improved the glomerular damage observed in ZO rats but did not ameliorate albuminuria, one can speculate that part of the albuminuria observed in ZO rats is due to a defect in proximal tubule reabsorption. In line with our present finding, Ac-SDKP did not reduce albuminuria in db/db mice, a mouse model of hyperinsulinemic diabetes with obesity (Shibuya et al., 2005). However, a separate study is required to understand the mechanism of albuminuria in these ZO rats.

We also showed that a HS diet increases the SBP only in ZO rats but not in ZL rats, confirming previous reports that ZO rats are salt sensitive (Ali et al., 2015; Reddy & Kotchen, 1992). We found that SBP was significantly increased in the ZO rats after 2 weeks of HS feeding (at 10 weeks of age) and that it remained elevated until the end of the 8 weeks of treatment (until 16 weeks of age) compared to the ZO rats fed a NS diet. Ac-SDKP treatment significantly reduced systolic blood pressure in the HS-fed ZO rats. In general, Ac-SDKP does not have any beneficial effect in lowering the blood pressure in various models of hypertension. (Liao et al., 2010; Rhaleb et al., 2011; Worou et al., 2015). However, recently, we have reported that Ac-SDKP delayed the onset of hypertension in an autoimmune model of systemic lupus erythematosus (Nakagawa et al., 2017). It is known that inflammation plays a role in blood pressure in various hypertension models (Harrison, Marvar, & Titze, 2012; Wenzel et al., 2011) and decreasing the inflammation reduces the elevated blood pressure (Guzik et al., 2007; Wenzel et al., 2011). Thus, in our study, the reduction in renal inflammation induced by Ac-SDKP could be a possible explanation for the decreased blood pressure in the HS-fed ZO rats.

We did not observe any change in the 24-hour sodium excretion in HS-fed ZL rats or HS-fed ZO rats with Ac-SDKP treatment. This observation eliminates the potential role of the lower

sodium intake in Ac-SDKP-treated animals. At the 8th week on HS, the blood pressure still tended to be lower in Ac-SDKP-treated ZO rats than in ZO rats without Ac-SDKP treatment; however, this decrement failed to reach statistical significance. In summary, our study provides evidence that a HS diet increased the renal damage (macrophage infiltration, fibrosis, and glomerulosclerosis) and blood pressure in ZO rats and that Ac-SDKP treatment prevented these changes without reducing albuminuria. Additionally, a HS diet per se was sufficient to exert mild renal inflammation in ZL rats. The HS diet increased glucose intolerance in ZO rats but the mechanism involved in this observation is not clear; however, studies from other labs have also shown similar findings (Donovan, Solomon, Seely, Williams, & Simonson, 1993; Ogihara et al., 2002).

We conclude that in HS-fed ZO rats, Ac-SDKP reduced renal inflammation and fibrosis and prevented/delayed the onset of hypertension.

Conflicts of Interest

The authors declared no conflict of interest.

Acknowledgments

This work was supported by the National Institutes of Health Grant P01HL028982 (O.A.C.)

CHAPTER 4

REGULATION OF GLOMERULAR CAPILLARY PRESSURE IN OBESITY: ROLE OF CONNECTING TUBULE GLOMERULAR FEEDBACK

This manuscript corresponds to Specific Aim 3 and deals with the hypothesis that increased CTGF contributes to TGF attenuation, which in turn increases P_{GC} in ZO rats.

Manuscript submitted to *American journal of physiology- Renal physiology*

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Abstract

Zucker obese (ZO) rats have higher glomerular capillary pressure (P_{GC}) which can cause renal damage. P_{GC} is controlled by the afferent (Af-Art) and efferent arteriole (Ef-Art) resistance. Af-Art resistance is regulated by factors that regulate other arterioles, such as myogenic response; in addition, it is also regulated by two intrinsic feedback mechanisms: 1) tubuloglomerular feedback (TGF) that causes Af-Art constriction in response to increased NaCl in the macula densa and 2) connecting tubule glomerular feedback (CTGF) that causes Af-Art dilatation in response to an increase in NaCl transport in the CNT *via* the epithelial sodium channel. Since CTGF is an Af-Art dilatory mechanism, we hypothesized that increased CTGF contributes to TGF attenuation, which in turn increases P_{GC} in ZO rats. We performed a renal micropuncture experiment and measured stop-flow pressure (P_{SF}), which is an indirect measurement of P_{GC} in ZO rats. Maximal TGF response at 40 nl/min was attenuated in ZO rats (4.47 ± 0.60 mm Hg) in comparison to the ZL rats (8.54 ± 0.73 mm Hg, $P < 0.05$), and CTGF was elevated in ZO rats (5.34 ± 0.87 mm Hg) compared to ZL rats (1.12 ± 1.28 mm Hg, $P < 0.05$). CTGF inhibition with epithelial sodium channel blocker normalized the maximum P_{SF} change in ZO rats indicating that CTGF plays a significant role in TGF attenuation (ZO, 10.67 ± 1.07 mm Hg vs. ZL, 9.5 ± 1.53 mm Hg). We conclude that enhanced CTGF contributes to TGF attenuation in ZO rats and potentially contributes to progressive renal damage.

Background

Obesity has become an epidemic worldwide and so has the increase in the obesity related renal damage. Almost 70% of the US population is overweight and among them more than 35% of the population is obese with a body mass index higher than 30 kg/m^2 (J. E. Hall et al., 2015). Furthermore, obesity is strongly associated with diabetes and hypertension, which are the two

top causes of end-stage renal failure (M. E. Hall et al., 2014; Leggio et al., 2017). Alterations in renal hemodynamics have been implicated as one of the key factors for the renal damage observed in obese individuals, but the mechanisms of the alterations in renal hemodynamics are unknown (Bondar et al., 2011; Bosma et al., 2006; Leggio et al., 2017). These hemodynamic changes include increased renal blood flow, glomerular capillary pressure (P_{GC}), and glomerular filtration rate (Bondar et al., 2011; Bosma et al., 2006; Sebekova et al., 2004). Sustained elevation in P_{GC} in particular can cause stretch in the glomerular cells and cause glomerular barotrauma that can lead to enhanced renal damage (Bondar et al., 2011; Riser et al., 1992; Sebekova et al., 2004).

In a normal kidney, renal blood flow is tightly controlled due to the existence of renal autoregulatory mechanisms that include tubuloglomerular feedback (TGF), connecting tubule glomerular feedback (CTGF), and myogenic response (Carlstrom et al., 2015; Monu et al., 2017). Afferent arterioles (Af-Art), glomerular capillaries, and efferent arterioles (Ef-Art) are arranged in series, and thus, their dynamics are closely interconnected (Monu et al., 2017). Arrangement of two resistance vessels, the Af-Art and the Ef-Art, regulate inflow and outflow of blood through the glomerular capillaries, and thus, regulate both P_{GC} and single nephron glomerular filtration rate (Monu et al., 2017). Af-Art constriction can reduce P_{GC} and glomerular plasma flow downstream that in turn can decrease glomerular filtration (Monu et al., 2017). Likewise, constriction of the Ef-Art can build the pressure upstream and may increase P_{GC} and single nephron glomerular filtration rate (Ren et al., 2001; H. Wang et al., 2015). Af-Art resistance is controlled by two renal intrinsic feedback mechanisms: 1) TGF that causes Af-Art constriction in response to increased NaCl in the macula densa, *via* the sodium–potassium-2-

chloride cotransporter-2, and 2) the CTGF that causes Af-Art dilatation and is initiated by the epithelial sodium channels (ENaC) in the CNT (Monu et al., 2017; H. Wang et al., 2015).

Previous studies suggest that there is increased P_{GC} (measured using the stop flow pressure method) in Zucker obese (ZO) rats (Park & Kang, 1995; Park & Meyer, 1995). TGF attenuation has been hypothesized for the enhanced pressure transmission from systemic circulation to the glomerulus leading to increased P_{GC}, but to our knowledge, no direct study has been done to evaluate the TGF mechanism in obesity. TGF attenuation could make the kidney susceptible to barotrauma and eventual glomerulosclerosis. However, the mechanism of enhanced P_{GC} in obesity is poorly defined.

Since CTGF is a vasodilator mechanism, we therefore investigated whether CTGF plays a role in TGF attenuation in obesity. We used ZO and Zucker lean (ZL) rats in our study. We hypothesized that increased CTGF contributes to TGF attenuation, which in turn increases P_{GC} in ZOR. To test this hypothesis, *in-vivo* renal micropuncture studies were performed in Zucker rats (8-10 week old) using the stop-flow technique.

Materials and Methods

Renal Micropuncture Experiment

The experiments were approved by Henry Ford Health System Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

We performed the renal micropuncture studies in ZO and ZL rats of 8-10 weeks of age, as described previously (H. Wang et al., 2015) (Figure 8). Briefly, rats were anesthetized with inactin intraperitoneally (125 mg/kg body weight for ZL rats and 175 mg/kg body weight for ZO rats). The left kidney was overturned and placed in a Lucite cup. Saline-soaked cotton was

placed around the kidney to immobilize it, and 30-45 minutes were allowed for equilibration. Colored dye was injected into surface tubules, permitting detection of tubule loops with a finding pipette. Grease was then injected into an early segment of the proximal tubule causing a tubule blockage, after which 2 pipettes were inserted inside the same tubule. First, a perfusion pipette was inserted downstream from the grease block and attached to the infusion pump. Second, a pipette for measuring P_{SF} was inserted upstream of the grease block and was attached to a micro-pressure system (model 900A; World Precision 99 Instruments, Sarasota, FL, USA). To generate a P_{SF} curve, the late proximal perfusion (in an orthograde manner) rate was increased stepwise from 0 to 10, 20, 30, and 40 nl/min while measuring P_{SF}. Each of the perfusion rates was maintained until we observed a stable P_{SF}. We performed two consecutive response measurements by perfusing the same tubule with vehicle and after with the ENaC inhibitor benzamil (1 μM).

TGF was calculated as a decrease in P_{SF} caused by an increase in nephron perfusion. CTGF was calculated as the difference between P_{SF} in the tubule perfused with vehicle and benzamil (1μM) as described previously (H. Wang et al., 2015). We also measured proteinuria (an early renal damage marker) in ZO rats.

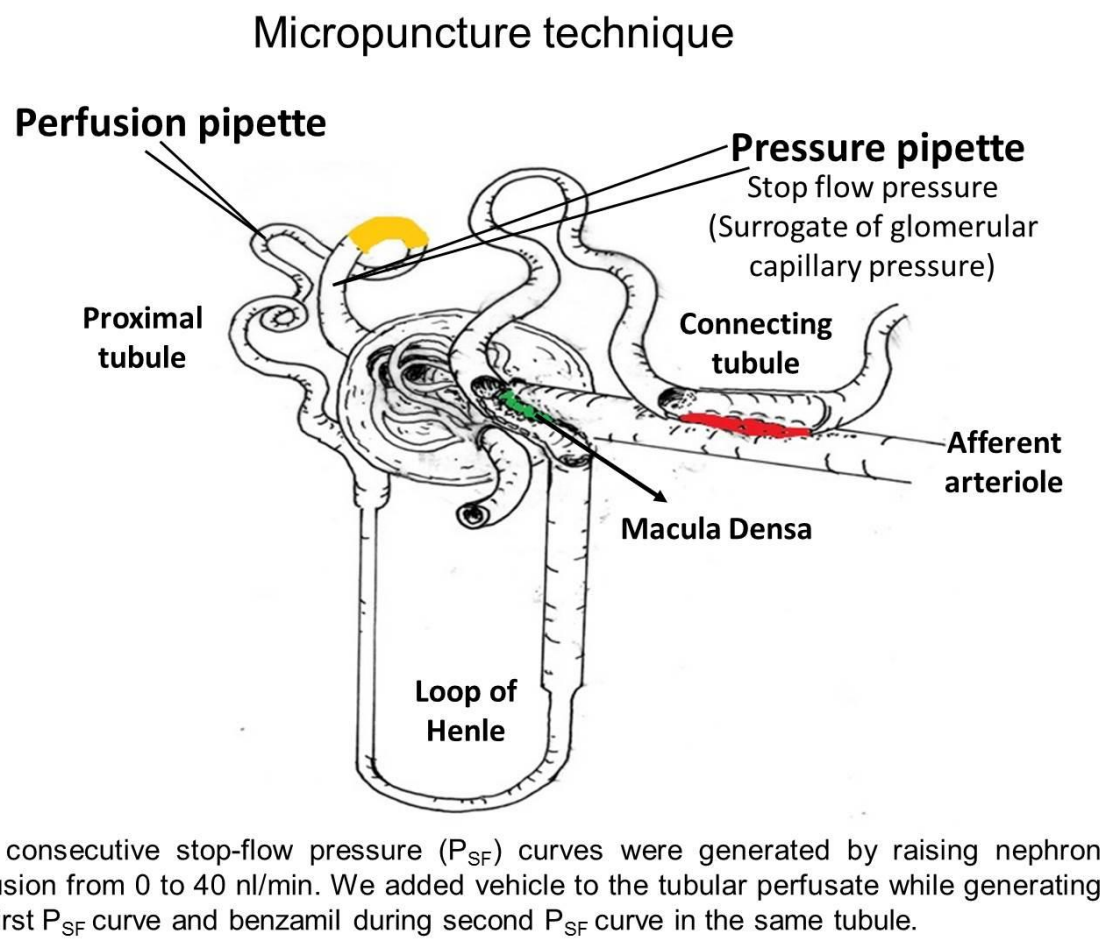

Figure 8. Schematic Diagram Explaining the Micropuncture Technique © Oscar A. Carretero.

Measurement of Proteinuria

After 24 hours of adaptation to metabolic cages, both ZL and ZO rats underwent urine collection for 24 hours. Total volume of collected urine was measured, aliquots prepared and centrifuged twice at 1200 g at 4ºC for 10 minutes. The supernatants were then filtered and stored at -80ºC until further analysis. Proteinuria was measured using a Coomassie Protein Assay Kit (Thermo Scientific, Massachusetts, USA) following the manufacturer's instructions. Proteinuria was calculated as urine protein concentration, respectively, multiplied by 24-hour urine volume output.

Statistical Analysis

Data are expressed as mean ± standard error. We used Student's two-sample t-tests, and for the measurement of repeated data, we used Student's paired t-tests. Hochberg's step-up procedure for adjusting p-values for multiple comparisons was used to control the family-wise type 1 error rate, predefined as 0.05.

Results

Time Control TGF Responses

To determine whether TGF responses varied with time, the late proximal tubule was perfused twice while measuring P_{SF}. During the experiment, the perfusion rate was increased from 0 to 40 nl/min step-wise at 10 nl/min intervals. We found no difference between the first and second curves in both ZL and ZO rats (Fig. 9A) and (Fig. 9B), indicating that this response was reproducible over time. We also observed that an increase in tubule perfusion decreased P_{SF} more in ZL than ZO rats.

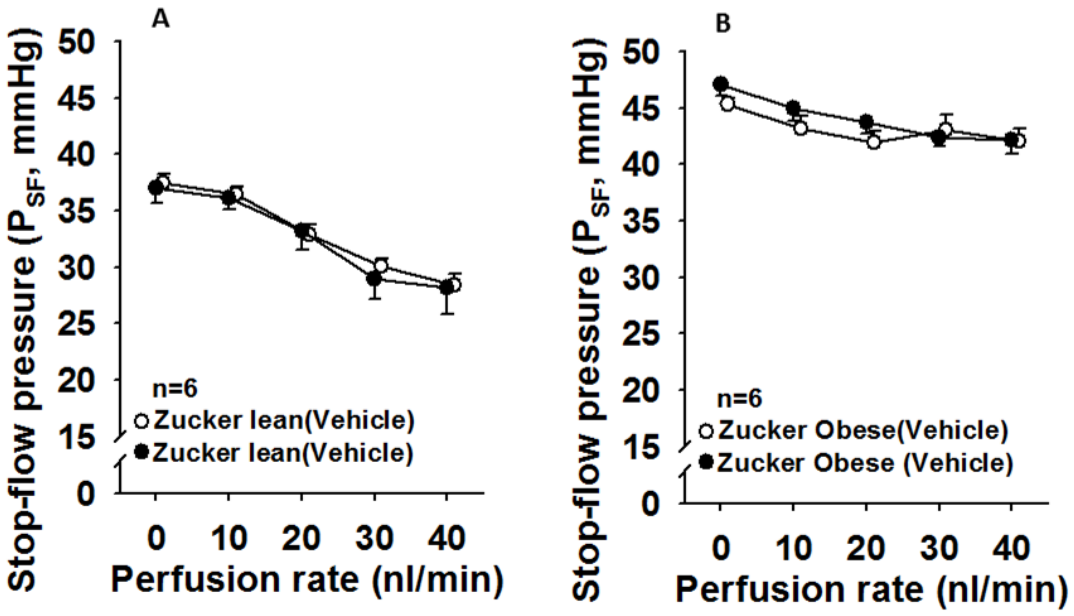

Figure 9. Time Control Experiments for ZL and ZO Rats. Increasing perfusion rates in the late proximal tubule two consecutive times does not affect P_{SF} reproducibly (○, first curve; •

second curve) in both ZL (A) and ZO rats (B). Both the curves were generated using same tubule. P_{SF}-Stop flow pressure, ZL-Zucker lean, ZO-Zucker obese

TGF Response (Maximum P_{SF} Change) is Decreased in ZO Rats

To determine whether obesity causes a decrease in the TGF response, maximum P_{SF} change (at 40 nl/min perfusion rate) was measured in both ZL and ZO rats. Maximum P_{SF} change was significantly lower ($P < 0.05$) in ZO compared to ZL rats. This result indicates that obesity causes TGF attenuation (Figs. 10A and 10B). We observed that P_{SF} was significantly higher in ZO compared to ZL rats.

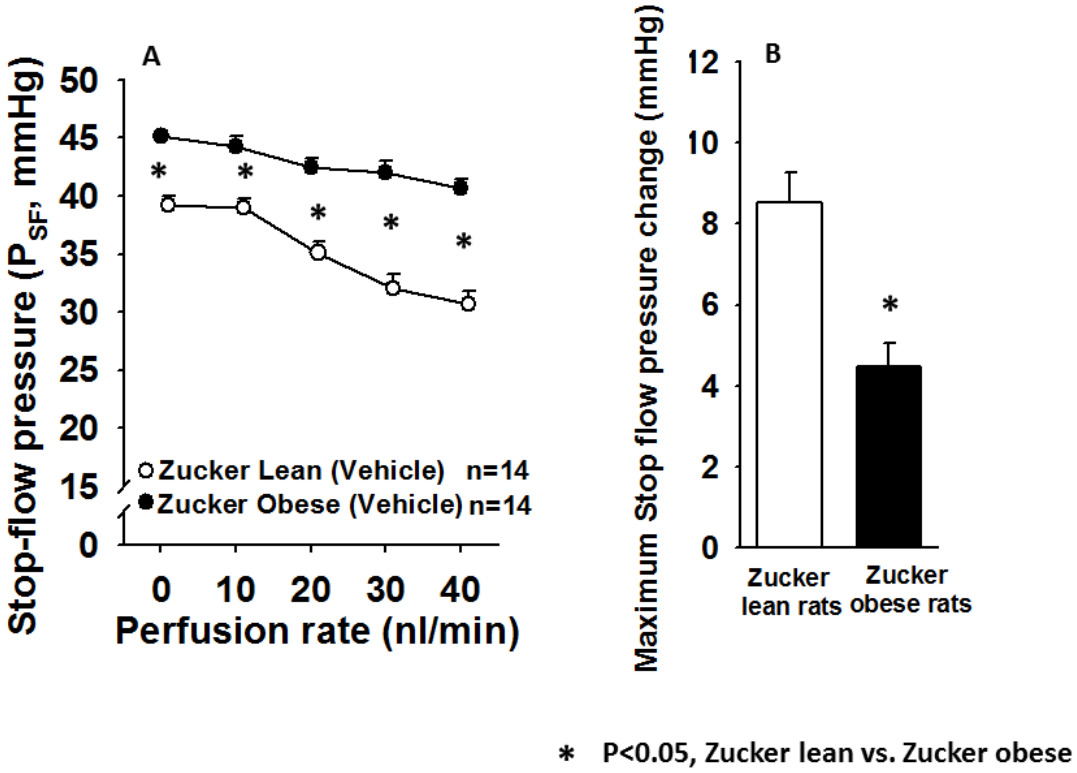

Figure 10. TGF Responses in ZL and ZO Rats. (A) TGF responses in ZL rats were significantly higher compared to ZO rats, indicating TGF attenuation. (B) Maximum P_{SF} change in ZL and ZO rats. *$P < 0.05$, ZL *vs*. ZO. P_{SF}-Stop flow pressure, TGF-Tubuloglomerular feedback, ZL-Zucker lean, ZO-Zucker obese

Benzamil Reduces P_{SF} in ZO Rats

To study the CTGF response in both ZL and ZO rats, we generated 2 consecutive P_{SF} curves from the same nephron tubule. The first P_{SF} response shows perfusion treatment with vehicle and the second with the ENaC blocker benzamil. To calculate CTGF, we subtracted the vehicle treatment P_{SF} values from benzamil treatment for each perfusion rate. The P_{SF} response during vehicle treatment was similar to the benzamil treatment in ZL rats. This result indicates that basal CTGF is absent in ZL rats (Fig. 11A).

We performed a similar experiment with intratubular vehicle and benzamil in ZO rats to evaluate the CTGF response. Benzamil treatment significantly increased the change in P_{SF} compared to the vehicle treatment at the 40nl/min perfusion rate indicating presence of CTGF in ZO rats (Fig. 11B).

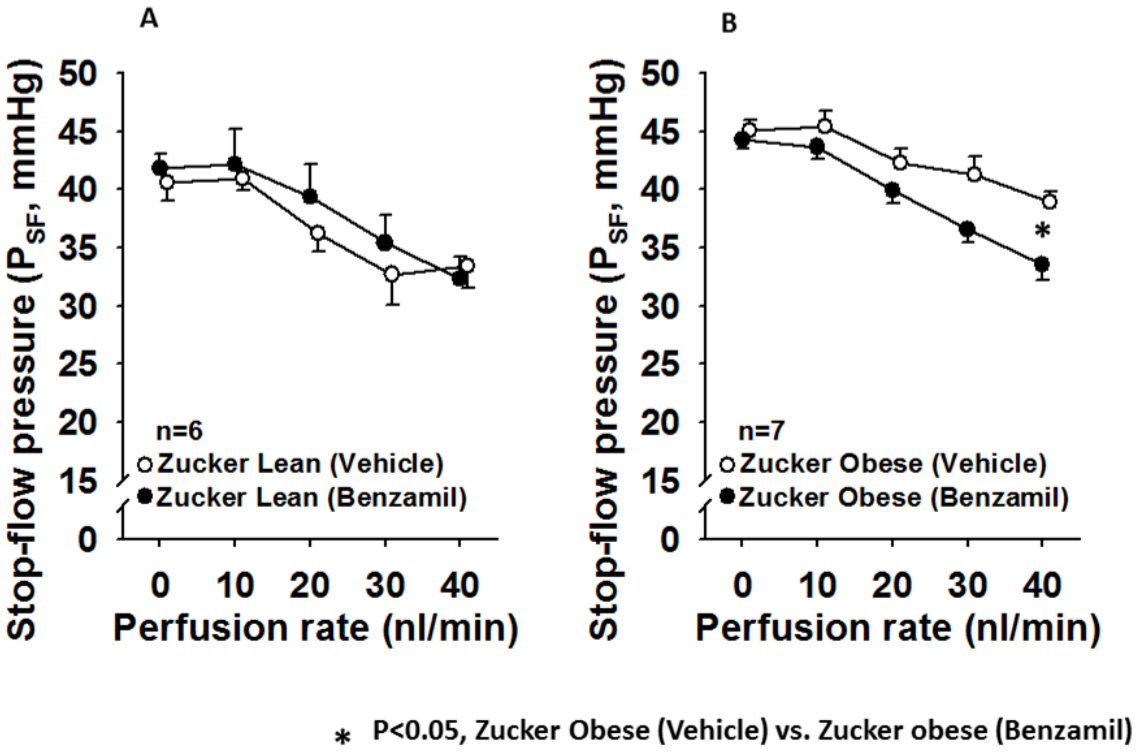

Figure 11. Effect of Benzamil on P_{SF} Change in ZL and ZO Rats. (A) ZL and (B) ZO rats *P < 0.05, ZO vehicle *vs*. ZO benzamil. P_{SF}-Stop flow pressure. ZL-Zucker lean, ZO-Zucker obese

CTGF Response in ZL and ZO Rats

To compare the CTGF response between ZL and ZO rats, we calculated the CTGF response in both ZL and ZO rats by subtracting the vehicle P_{SF} values from the P_{SF} values obtained after intratubular benzamil treatment at each perfusion rate. The CTGF value at 40 nl/min was significantly enhanced ($P < 0.05$) in ZO compared to ZL rats (Fig.12).

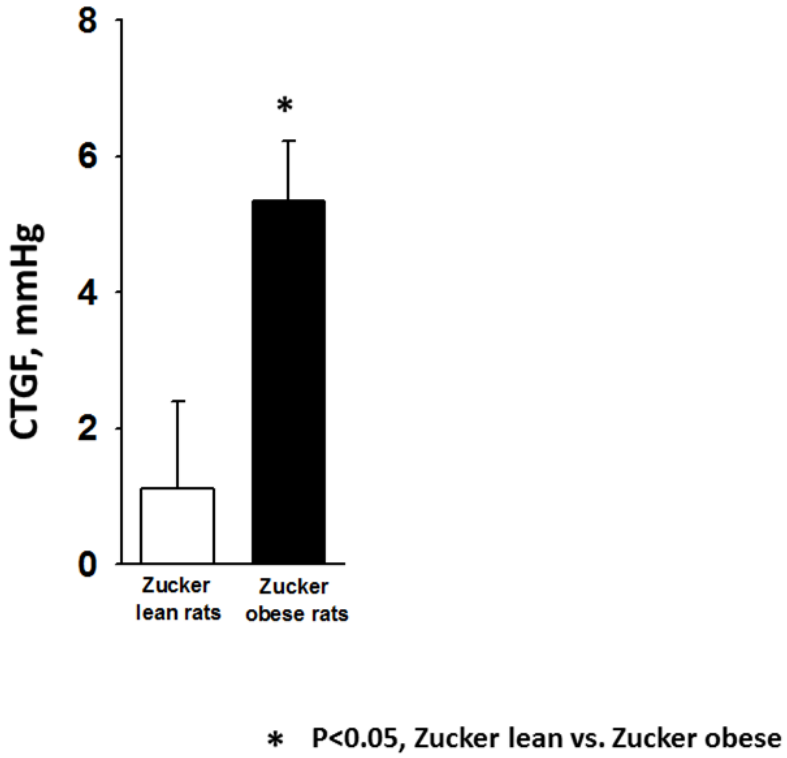

Figure 12. Comparison of Maximum CTGF in ZL and ZO Rats. CTGF is enhanced in ZO (black bar) compared to ZL (open bar) *$P < 0.05$, ZL *vs.* ZO. CTGF-Connecting tubule glomerular feedback, ZL-Zucker lean, ZO-Zucker obese

Absolute TGF Response After Benzamil Treatment in ZL and ZO Rats

In the presence of benzamil, P_{SF} decreased in response to the increase in the nephron perfusion rate in both ZL and ZO rats (Fig.13). This decrease in P_{SF} upon benzamil treatment was similar in ZL and ZO rats. These data also show that upon CTGF inhibition, TGF behaves similarly in both of the ZL and ZO rat groups.

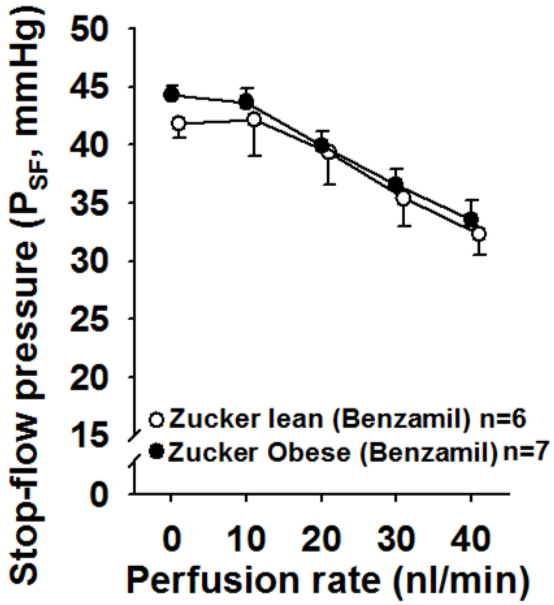

Figure 13. Effect of Benzamil on P_{SF} in ZL and ZO Rats. Inhibition of CTGF eliminates the decrease in TGF in the ZO (○, ZL benzamil; • ZO benzamil). CTGF-Connecting tubule glomerular feedback, P_{SF} -Stop flow pressure ZL-Zucker lean, ZO-Zucker obese

Measurement of Renal Damage in Zucker Rats

Proteinuria was found to be significantly elevated starting from the age of 12 weeks and onwards in ZO compared to ZL rats, indicating higher renal damage in ZO rats (Fig.14).

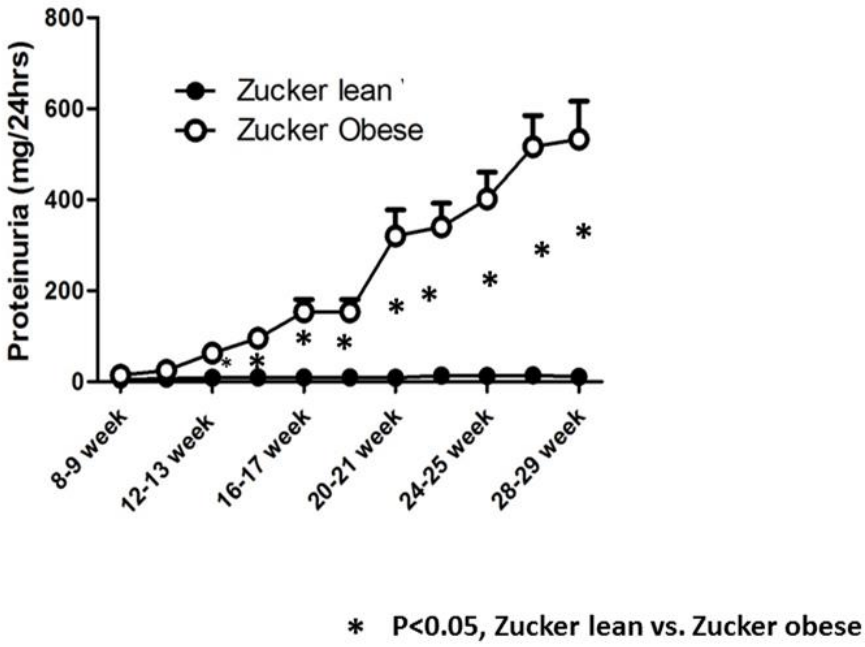

Figure 14. Measurement of Renal Damage in Zucker Rats. Proteinuria was found to be elevated starting from the age of 12 weeks and onwards in ZO compared to the ZL rats indicating higher renal damage in obesity. ZO (open circle) and ZL (closed circle), *P<0.05, ZO *vs* ZL rats.

Mean Arterial Pressure and Body Weight in Zucker Rats

Mean arterial pressure measured during renal micropuncture via intrafemoral arterial catheter under anesthesia was found to be mildly elevated in ZO compared to ZL rat controls. Additionally, ZO rats weighed significantly higher compared to ZL controls (Fig.15).

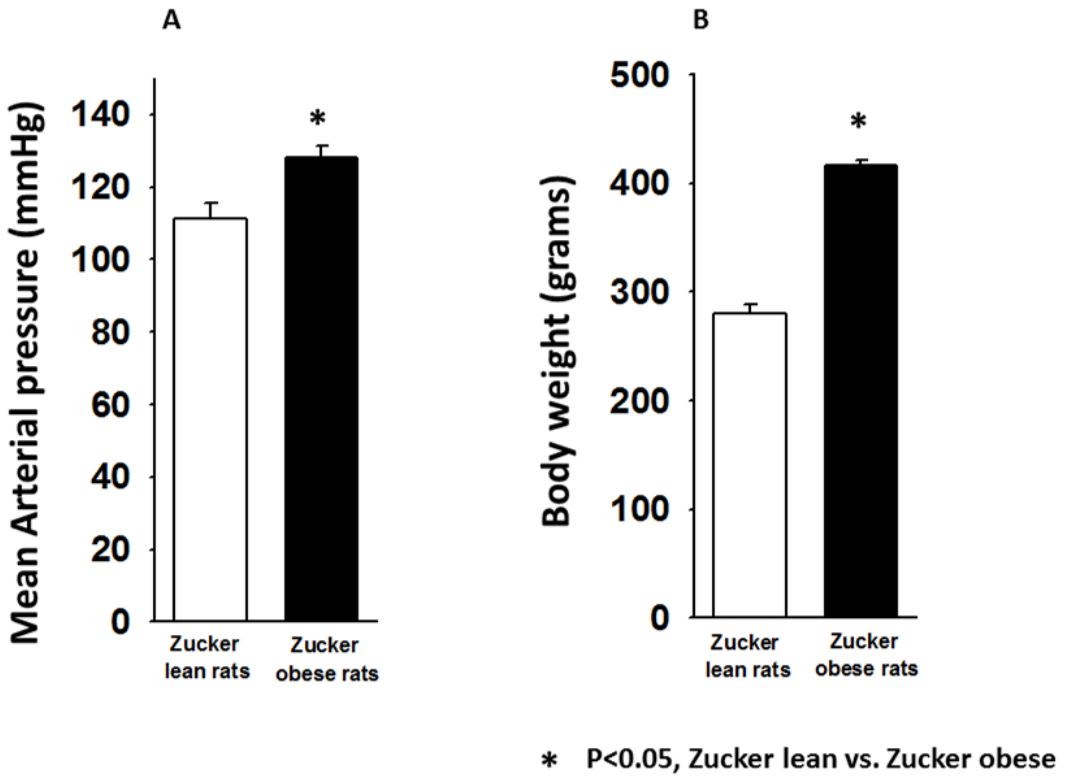

Figure 15. Blood Pressure and Body Weight in Zucker Rats. Mean arterial pressure measured during renal micropuncture via intra-femoral arterial catheter under anesthesia was found to be elevated in ZO rats compared to the lean controls (Fig. 15 A). Additionally, ZO rats weighed significantly higher compared to the lean controls (Fig.15 B). Mean arterial pressure (A) and body weight (B) in ZL and ZO rats. ZL (open bar) and ZO (closed bar). *P<0.05, ZO *vs*. ZL rats.

Discussion

In this study, we investigated whether renal intrinsic feedback mechanisms (TGF and CTGF) play any role in the regulation of P_{GC} in ZO rats. Our results show that in ZO rats, TGF was attenuated and CTGF was enhanced. We also observed higher P_{GC} in ZO rats at 8-10 weeks of age, which was followed by proteinuria at 12 weeks of age.

Our first finding that ZO rats have higher P_{GC} is supported by our renal micropuncture results wherein we measured the P_{SF}, a surrogate of P_{GC}. In 8-10 week old Zucker rats, we found that P_{SF} was significantly higher in ZO when compared to ZL rats (Fig. 10A). This finding is similar to the finding of Park and Kang wherein they found P_{GC} to be higher in ZO rats (Park &

Kang, 1995). ZO rats undergo numerous hemodynamic changes that include elevated renal blood flow, $P_{GC,}$ and single nephron glomerular filtration rate. Elevated P_{GC} has been implicated in causing glomerular barotrauma leading to renal damage in various renal disease models (Monu et al., 2017; H. Wang, D'Ambrosio, Garvin, Ren, & Carretero, 2013). Our results show that ZO rats develop proteinuria at the age of 12 weeks and that it gets exaggerated with age (Fig. 14). Others have also shown ZO rats having higher renal damage when compared to ZL controls. Also, in humans, it has been reported that obesity increases the risk of renal damage and humans frequently become proteinuric (Kovesdy et al., 2017; Praga & Morales, 2006; Yamahara et al., 2013).

Previously, we described renal autoregulatory mechanism acting opposite of TGF at the single-nephron level, called CTGF (Monu et al., 2017; Ren, D'Ambrosio, Garvin, Wang, & Carretero, 2013). In opposition to TGF, CTGF is a vasodilator mechanism initiated in the CNT segment of the nephron *via* ENaC (Monu et al., 2017). In the current study, we found that compared to ZL rats, TGF in ZO rats are significantly attenuated (Figs. 10A and 10B). To our knowledge, this is the first study to report an attenuated TGF response in ZO rats. Our current data show that intratubular inhibition of CTGF with benzamil significantly reduces P_{SF} in ZO rats, but it remains unchanged in ZL rats. These data suggest that TGF resetting in ZO rats is due to enhanced CTGF.

In the ZO rats group, intratubular perfusion of benzamil restored the TGF response to a level similar to the ZL rats group (Fig. 13). Thus, these data may suggest that upon blocking CTGF, TGF could become operational in ZO rats. Obesity seems to shift the balance between vasoconstrictive (TGF) and vasodilatory (CTGF) mechanisms in favor of the latter, resulting in

high $P_{SF.}$ Enhanced CTGF may also be responsible for the increased glomerular plasma flow rate observed in ZO rats (Park & Kang, 1995) due to its Af-Art dilator mechanism.

The mechanism by which CTGF is enhanced in ZO rats remains elusive. We know that CTGF is initiated by sodium transport in the CNT *via* ENaC. In ZO rats, the expression and activity of ENaC channels are enhanced (Bickel, Verbalis, Knepper, & Ecelbarger, 2001; Madala Halagappa, Tiwari, Riazi, Hu, & Ecelbarger, 2008). Earlier, we have shown that the effect of CTGF is mediated by prostaglandin E2 (Ren et al., 2013) and epoxyeicosatrienoic acid (Ren, D'Ambrosio, et al., 2009). Prostaglandin binds on the prostaglandin E2 receptor 4 on the Af-Art and causes dilation, thus, eliciting CTGF (Ren et al., 2013).

Prostaglandins and epoxyeicosatrienoic acid may be playing a role for the mediation of CTGF in these ZO rats. Furthermore, it has been shown that cyclooxygenase-2 (precursor of prostaglandins) expression is enhanced in the kidney of ZO rats (Komers et al., 2005).

In our study, we found that P_{SF} is significantly increased in ZO rats in comparison to ZL controls even when the perfusion rate was zero; i.e., in the absence of TGF and CTGF. This increased P_{SF} could be due to reduced myogenic response, elevated mean arterial pressure or constriction of Ef-Art in these ZO rats. In fact, earlier studies have shown that the myogenic response is significantly reduced in ZO rats (Hayashi et al., 2002). Another possibility of increased basal P_{GC} could be due to enhanced Ef-Art constriction. Direct measurement of Ef-Art resistance in ZO rats has been reported to be higher (Roos et al., 2008). Moreover, in-vitro experiments suggest a vasodilatory effect of TGF on the Ef-Art; thus, one may speculate that in ZO rats, in which TGF is attenuated, the Ef-Art may constrict, resulting in elevation of P_{GC} (Ren, Garvin, Liu, & Carretero, 2007).

In the current study, we have indirectly measured the P_{GC} as P_{SF} due to a lack of surface glomeruli in these rats. To our knowledge, nobody has reported the direct measurement of P_{GC} in these ZO rats. Additionally, we have not measured the single nephron glomerular filtration rate in our study and apart from an increase in the P_{GC}, increased single nephron glomerular filtration rate, increased mean arterial pressure (Fig 15) and elevated single nephron plasma flow per se could be involved in playing a role in renal damage (Fig 14) in this obese model.

In summary, our studies provide direct evidence of TGF resetting in ZO rats and these differences are attributed at least in part due to enhanced CTGF. ZO rats also showed higher P_{GC} followed by higher renal damage compared to the ZL controls. Increased CTGF may help explain the increased P_{GC}, reduced TGF response and increased renal damage in ZO rats.

Acknowledgments

We thank Carl Polomoski for the technical assistance in this project. We also thank Stephanie Stebens for the grammar correction of the manuscript.

Conflicts of Interest

The authors declared no conflicts of interest.

Source of Funding

Research reported in this publication was supported by the Heart, Lung, and Blood Institute of the National Institutes of Health under Award number HL-028982. The content is solely the responsibility of the authors, and does not necessarily represent the official views of the National Institutes of Health.

CHAPTER 5

SUMMARY, CONCLUSIONS AND FUTURE PROSPECTS

This research study is divided in two parts. In the first part we studied the renoprotective role of an anti-inflammatory peptide known as Ac-SDKP in obesity induced renal damage, and in Part 2 we studied the role of enhanced connecting tubule glomerular feedback mechanism in obesity induced renal damage.

Obesity is linked to salt sensitive hypertension and renal damage. In obesity, there is inflammation of kidney that eventually leads to fibrosis. Ac-SDKP is a naturally occurring tetrapeptide that has been shown to have anti-inflammatory and anti-fibrotic properties in various models of cardiovascular and renal diseases but its effect on obesity induced renal disease is not known. The present study demonstrated that Ac-SDKP treatment prevented high salt induced hypertension and renal damage in Zucker obese Rats. We found that high salt diet significantly increased the BP in ZO rats and Ac-SDKP significantly reduced the elevated blood pressure in ZO rat. Infiltration of macrophages plays an important role in causing obesity related renal damage. Our data suggested that macrophage infiltration was significantly increased in ZO rats with high salt diet and Ac-SDK attenuated this increase. Inflammation eventually leads to fibrosis; we observed that both cortical and medullary interstitial fibrosis was significantly increased in ZO rats with high salt. Ac-SDKP could prevent this increase. In line with this, we found increased glomerulosclerosis in ZO rats on high salt and Ac-SDKP treatment significantly reduced the glomerulosclerosis in these rats.

Alterations in renal hemodynamics such as increased renal blood flow, and enhanced glomerular capillary pressure have been implicated both clinically as well as in experimental settings as one of the key factors for the renal damage observed in obesity, but the mechanism of

the alterations in renal hemodynamics is largely unknown. Here we show that ZO rats have higher glomerular capillary pressure (P_{GC}) that is followed by enhanced renal damage. P_{GC} is normally controlled by the afferent (Af-Art) and efferent arteriole (Ef-Art) resistance. Af-Art resistance is regulated by two intrinsic feedback mechanisms: 1) tubuloglomerular feedback (TGF) that causes Af-Art constriction in response to increased NaCl in the macula densa and 2) connecting tubule glomerular feedback (CTGF) that causes Af-Art dilatation in response to an increase in NaCl transport in the CNT via the epithelial sodium channel. In our present study, we observed an enhanced CTGF that contributes to TGF attenuation in ZO rats. This enhanced CTGF may lead to increased dilation of afferent arteriole and thus enhanced systemic pressure transmission to the glomerulus leading to kidney damage in ZO rats.

To summarize, enhanced systemic pressure transmission to glomerulus may lead to glomerular barotrauma that in turn may initiate proteinuria and the subsequent inflammation cascade leading to renal damage in obesity (Figure 16)

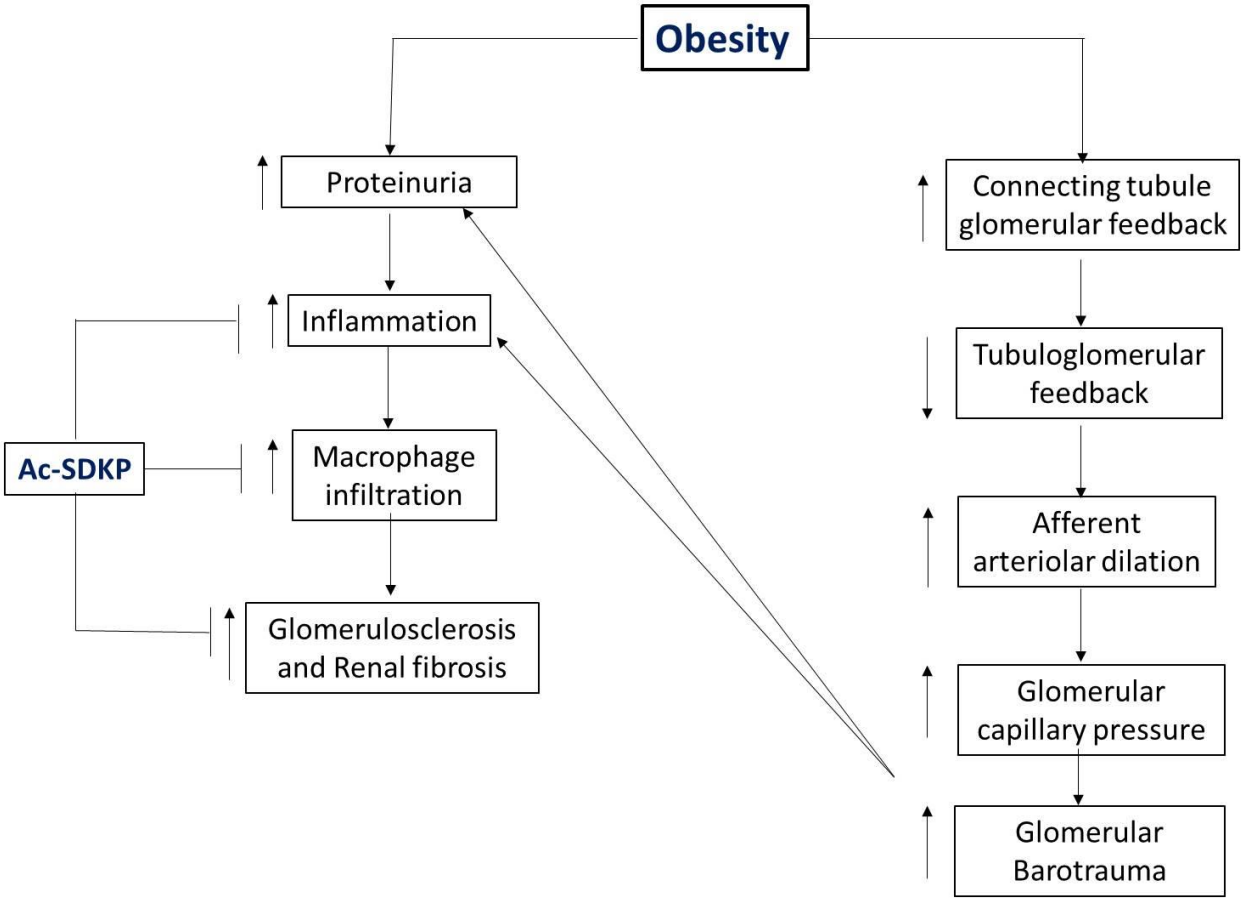

Figure 16. Scheme Showing the Mechanisms of Renal Damage in Obesity and Effect of Ac-SDKP on Preventing the Damage.

Conclusions

Based on the findings of the first part of the present study, we conclude that Ac-SDKP treatment in ZO rats fed a high salt diet prevented renal damage by reducing fibrosis, inflammation and blood pressure.

For Part 2 we conclude that enhanced CTGF contributes to TGF attenuation In ZO rats and potentially contributes to progressive renal damage.

Future Prospects

Ac-SDKP has been shown to improve the renal damage in various models of renal dysfunction, but for the first time in this study, Ac-SDKP decreased blood pressure. The possible role of Ac-SDKP in ameliorating the blood pressure could be of immense importance and require

further studies. The mechanism involved in ameliorating the blood pressure by Ac-SDKP will be an interesting area of future research which could bring new advancements in the field of hypertension. Additionally, the long term effects of Ac-SDKP as treatment for renal damage should be investigated.

Our pre-clinical study demonstrates that high salt diet could exert harmful effects not only in obese individuals, but also in non-sensitive individuals and that Ac-SDKP exerts strong renal protective effects. Angiotensin converting enzyme inhibitors (ACEi) are widely used in treating obesity related kidney damage and hypertension; however, some patients cannot tolerate ACEi-associated side effects such as hypotension, hyperkalemia, and angioedema. Thus, Ac-SDKP or its analog, resistant to enzymatic degradation, could be a novel and useful therapeutic strategy for treating high salt-induced obesity related renal damages.

Additionally, inhibiting CTGF may reduce glomerular barotrauma and thus prevent glomerular damage and its sclerosis.

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APPENDIX A: OFFICE OF RESEARCH INTEGRITY APPROVAL LETTER

MARSHALL UNIVERSITY.
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Office of Research Integrity

May 9, 2018

Mani Maheshwari
Hypertension and Vascular Research Division
2799 W. Grand Blvd.
E&R Bldg., Room 7017
Henry Ford Hospital
Detroit, MI 48202

Dear Mani:

This letter is in response to the submitted thesis abstract entitled *"Role of N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) and renal hemodynamics on obesity related renal damage."* After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Institutional Animal Care and Use Committee (IACUC) of Henry Ford Hospital has reviewed and approved the study. The applicable human and animal federal regulations have set forth the criteria utilized in making this determination. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director

APPENDIX B: LIST OF ABBREVIATIONS

α-SMA	Alpha smooth muscle actin
ACE	Angiotensin converting enzyme
ACEi	Angiotensin converting enzyme inhibitor
Ac-SDKP	N-acetyl-seryl-aspartyl-lysyl-proline
Af-Art	Afferent arteriole
ARB	Angiotensin Type 1 receptor blocker
Ang II	Angiotensin II
AT1	Angiotensin 1
AT2	Angiotensin 2
AUC	Area under curve
BBB	Blood brain barrier
BMI	Body mass index
BW	Body weight
CD2AP	CD2-associated protein
CKD	Chronic kidney disease
CNT	Connecting tubule
CTGF	Connecting tubule glomerular feedback
DCT	Distal convoluted tubule
DOCA	Deoxycorticosterone acetate
ECM	Extracellular matrix
Ef-Art	Efferent arteriole
ELISA	Enzyme linked immunosorbent assay

EMT	Epithelial-mesenchymal transition
ENaC	Epithelial sodium channel
EndMT	Endothelial-mesenchymal transition
EP4	Prostaglandin E2 receptor 4
ERK1/2	Extracellular signal-regulated kinase 1/2
FGF	Fibroblast growth factor
FSGS	Focal segmental glomerulosclerosis
GFR	Glomerular filtration rate
GTT	Glucose tolerance test
HS	High-salt
IL	Interleukin
ipGTT	Intraperitoneal glucose tolerance test
LV	Left ventricle
MCP-1	Monocyte chemoattractant protein 1
MiR	MicroRNA
MMP	Matrix metalloproteinase
MR	Mineralocorticoid receptor
MRI	Magnetic resonance imaging
MWF	Munich wistar fromter
NaCl	Sodium chloride
NCC	Sodium chloride cotransporter
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells

NKCC2	Sodium-potassium-2-chloride cotransporter-2
NHE	Sodium hydrogen exchanger
NS	Normal-salt
PAS	Periodic-acid Schiff
PAI-1	Plasminogen activator inhibitor-1
PDGF	Platelet-derived growth factor
PG	Prostaglandin
P_{GC}	Glomerular capillary pressure
POP	Prolyl oligopeptidase
PPAR γ	Peroxisome proliferator-activated receptor gamma
PPRE	Proliferator activated response element
PSR	Picrosirius red
P_{SF}	Stop flow pressure
PT	Proximal tubule
RAS	Renin angiotensin system
RAAS	Renin-angiotensin-aldosterone system
ROS	Reactive oxygen species
RBF	Renal blood flow
SBP	Systolic blood pressure
SGK1	Glucocorticoid-inducible-kinase 1
SHR	Spontaneously hypertensive rat
SLE	Systemic lupus erythematosus
SNS	Sympathetic nervous system

SPAK	SPS-1 related proline/alanine-rich kinase
STZ	Streptozotocin
TBI	Traumatic brain injury
Tβ4	Thymosin beta 4
TGF	Tubuloglomerular Feedback
TGFβ	Transforming growth factor beta
TIMP	Tissue inhibitor of metalloproteinase
TNF-α	Tumor necrosis factor alpha
tPA	Tissue plasminogen activator
UUO	Unilateral ureter obstruction
WHR	Waist to hip ratio
WNK-4	With-no-lysine kinase-4
ZL	Zucker lean
ZO	Zucker obese

APPENDIX C: VITA

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Education

July 2013-2018	Doctor of Philosophy in Biomedical Sciences, Marshall University School of Medicine, Huntington, WV
December 2008- August 2010	Masters in Veterinary Public Health, College of Veterinary Science, Rajendra Nagar Hyderabad, Andhra Pradesh, India
August 2003- October 2008	Bachelors in Veterinary Sciences and Animal Husbandry, Madras Veterinary College, Chennai, Tamil Na

Research

2013-Present	Graduate Research Assistant Department of Physiology, Pharmacology and Toxicology, Marshall University, West Virginia. USA
February 2011-January 2012	Research Associate, Central Research institute for Dryland Agriculture, Hyderabad, India
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Peer Reviewed Publications

Mani Maheshwari, Cesar A Romero, Sumit R Monu, Nitin Kumar, Tang-Dong Liao, Edward L Peterson, Oscar A Carretero (2018). Renal Protective Effects of N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) in Obese Rats on a High-Salt Diet. *Am J Hypertens.*

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Abstracts and Presentations

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Awards

Onsite Trainee Poster Award from American Heart Association (2017), Council on Hypertension Trainee Advocacy Committee and the International Society of Hypertension New Investigator Committee, San Francisco.

University Gold Medal (2010) - for highest OGPA in Masters of Veterinary Public Health. College of Veterinary Science, Hyderabad, India.

Gold Medal from Dr. C Krishna Rao Endowment Trust (2010) for being the topper in the University in Masters of Veterinary Sciences (M.V.Sc.) in Veterinary Public health.