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# Role of Angiotensin II Type 1A Receptors on Renal and Urinary Angiotensin Converting Enzyme 2 (ACE2) and Neprilysin (NEP) in the Two-Kidney One-Clip (2K1C) Model of Renovascular Hypertension

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# Role of Angiotensin II Type 1$_A$ Receptors on Renal and Urinary Angiotensin Converting Enzyme 2 (ACE2) and Neprilysin (NEP) in the Two-Kidney One-Clip (2K1C) Model of Renovascular Hypertension

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Master of Science

By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY LAALE F. ALAWI ENTITLED "ROLE OF ANGIOTENSIN II TYPE $1_A$ RECEPTORS ON RENAL AND URINARY ANGIOTENSIN CONVERTING ENZYME 2 (ACE2) AND NEPRILYSIN (NEP) IN TWO-KIDNEY ONE-CLIP (2K1C) MODEL OF RENOVASCULAR HYPERTENSION" BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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# ABSTRACT

Alawi, Laale Fakhri, M.S, Department of Pharmacology and Toxicology, Wright State University, 2015. Role of Angiotensin II Type $1_A$ Receptors on Renal and Urinary Angiotensin Converting Enzyme 2 (ACE2) and Neprilysin (NEP) in the Two-Kidney One-Clip (2K1C) Model of Renovascular Hypertension

Activation of the renin angiotensin system (RAS) and increased formation of angiotensin (Ang) II contribute to the progression of chronic kidney disease (CKD). Ang II, the major biologically active peptide of RAS, acts mainly as a vasoconstrictor through binding to the Ang II type 1 receptor (AT1R), which leads to increased blood pressure, fluid retention, and aldosterone secretion. The actions of Ang II are antagonized by its conversion to the vasodilator Ang (1-7), partly generated by the action of angiotensin converting enzyme 2 (ACE2) and/or neprilysin (NEP). The metalloprotease ADAM17 has a crucial role in the shedding of renal ACE2 in diabetic mice model. The two-kidney, one clip (2K1C) Goldblatt model is an experimental approach designed to mimic renovascular hypertension. It consists of the unilateral clamping of the renal artery in one of the kidneys. The aim of this study is to test the hypotheses that: 1) renovascular hypertension and increased albuminuria in the 2K1C model is mediated by $AT1_A R$ and 2) up-regulation of renal ADAM17 increase the shedding of renal ACE2 and NEP into the urine. Wild type (WT) and $AT1_A R$ knockout (AT1 KO) mice were used to test our hypotheses. Mice were subjected to surgical procedures to implant radio-telemetry transmitters for measurement of blood pressure (BP), followed by induction of renovascular hypertension. BP at baseline was significantly lower in AT1 KO compared to WT mice, whereas in WT 2K1C, BP was significantly higher than controls ($p<0.05$). However, 2K1C has no effect on BP in AT1 KO mice. Urinary albumin excretion significantly increased in WT 2K1C mice compared to sham -operated ones, while no change was observed in AT1 KO. In addition, a significant reduction of renal ADAM17 and NEP contents was observed in clipped kidney relative to the unclipped and sham kidneys. Western blot analysis

showed a significant decrease in renal ACE2, NEP, and ADAM17 protein expression levels in the clipped kidney compared to the unclipped or sham-operated ones. Histological assessment of the kidneys in the 2K1C model revealed significant mesangial expansion and renal fibrosis. Data suggest that renovascular hypertension is mediated by $AT1_AR$ and deletion of this receptor attenuates albuminuria in the 2K1C model. In 2K1C, the downregulation of renal and urinary ACE2 and ADAM17 suggest a potential link between ADAM17 and ACE2 shedding in 2K1C mice. Decreased renal NEP in the clipped kidney of 2K1C model may thus worsen kidney injury via impairment of Ang (1-7) formation.

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# 1. INTRODUCTION

## 1.1. Hypertension

High blood pressure is one of the major common risk factors leading to morbidity and mortality, due to its role in the development of several disorders, including heart failure, cardiac hypertrophy, stroke, and chronic kidney disease (CKD) (Chen & Coffman, 2012). Hypertension is a chronic condition that affects around 25% of the total adult population worldwide, and is predicted to increase to 29% by the year 2025 (Kearney et al., 2004). A recent study shows a link between metabolic syndrome and CKD, in which hypertension plays a major role leading to the end stage renal disease (ESRD) when compared to other metabolic syndromes (Nashar & Egan, 2014). Hypertension is defined by the guidelines of the Joint National Committee on detection, evaluation, and treatment of high blood pressure. It consists three stages as shown in Table 1 (Chobanian et al., 2003):

**Table 1: Classification of hypertension**

| Classification | Systolic BP (mmHg) | Diastolic BP (mmHg) |
|---|---|---|
| Normal | <120 | <80 |
| Pre-hypertension | 120-139 | 80-89 |
| Stage 1 hypertension | 140-159 | 90-99 |
| Stage 2 hypertension | ≥160 | ≥100 |

If systolic blood pressure (SBP) is more than 180 mmHg, or diastolic blood pressure (DBP) more than 110 mmHg, emergency care is needed. These cases are considered stage 3 hypertension, also known as hypertensive crisis (Lackland, 2013). According to the 2014-hypertension guidelines, the target blood pressure in a general population aged ≥60

years should be 150/90 mmHg, whereas the target blood pressure should be 140/90 mmHg

for a population younger than 60 years and with diabetes or CKD, (James et al., 2014).

## 1.2. Role of the kidney in hypertension

The link between kidney and blood pressure control has been identified in the 19th century

by Richard Bright, followed by Goldblatt's report who developed a renovascular

hypertension animal model by narrowing one of the renal arteries, known as the Goldblatt

hypertension model (Goldblatt et al., 1934). It is well known that essential hypertension

could be effected either by renal abnormalities such as a decrease of kidney function or by

over activation of the renin angiotensin system (Hall et al., 1996). Activation of the

intrarenal renin angiotensin aldosterone system (RAS) plays a crucial role in the

progression of hypertension primarily through the increase of Ang II and its type 1

receptor (Kobori et al., 2007). Reduced blood pressure may occur through inhibition of

specific RAS components such as renin, angiotensin converting-enzyme, and angiotensin

II type 1 receptor (Crowley et al., 2007).

## 1.3. Pathophysiology of renovascular hypertension (RVH)

Renovascular hypertension (RVH) occurs when main renal arteries are obstructed,

resulting in an increase in the systemic blood pressure (Textor, 2009).The essential role of

RAS has been confirmed in the development of high blood pressure in hypertensive

models (Cervenka et al., 2002; Navar et al., 1998). Emerging evidence demonstrates that

RVH is associated with ESRD when compared with the other causes of ESRD (Fatica et

al., 2001). Goldblatt and his colleagues were the first to establish the two classic Goldblatt

RVH animal models, the two-kidney one-clip (2K-1C) and the one-kidney one-clip (1K-

1C). Both models lead to hypertension, but with different mechanisms (Goldblatt et al., 1934).

## 1.4. Goldblatt renovascular hypertension models

### 1.4.1. One-kidney one clip (1K1C)

The activation of RVH in this model occurs via bilateral renal artery stenosis, by occluding the renal artery in one kidney, and removing the other kidney (Wiesel et al., 1997). Plasma renin elevation is caused by the reduction in renal arterial pressure, leading to an increase of Ang II in the circulation, which is considered an early phase of hypertension. These steps also occur in the 2K1C model; however, each model has different mechanisms. In 2K1C, presence of the contralateral non-clipped kidney keeps the RAS activated maintaining blood pressure and volume. However, the 1K1C model is considered a low renin volume dependent hypertension model, in which blockade of renin plasma secretion results in suppressed RAS activity (Wiesel et al., 1997).

### 1.4.2. Two-kidney one clip (2K1C)

Goldblatt originally developed this model using dogs to study the pathogenesis of RVH (Goldblatt et al., 1934). The model was extended to other species such as rats, mice, and pigs (Thone-Reineke et al., 2003). In this model, RVH is mediated by a unilateral renal artery stenosis, leading to a reduced renal perfusion in the clipped kidney, and activation of the Ang II that increased the release of renin from the kidney (Campagnaro et al., 2013). The 2K1C model recently helped to determine several factors that might play a role in the development of cardiovascular and renal diseases (Al-Suraih & Grande, 2014); one of which is the activation of RAS that mediates the vasoactive effect. The 2K1C model is also categorized as an Ang II-dependent hypertension model, in which increased renin

release leads to elevated renal Ang I, Ang II, and ACE resulting in a further increase in blood pressure (Prieto et al., 2011). In addition, increased ACE in the 2K1C Goldblatt hypertensive model has a reciprocal association with Ang (1-7), ACE2, and neprilysin (NEP) in the affected kidney and thoracic aorta (Jongun, 2004). A study by Oliveira Sales et al. proposed that angiotensin II type 1 receptor (AT1R) and inducible nitric oxide synthase (iNOS) in the kidney, specifically in the rostral ventrolateral medulla, controls hypertension and renal sympathetic activation in the 2K1C model (de Oliveira-Sales et al., 2010). Recently, mesenchymal stem cells show a beneficial effect in the treatment of renovascular hypertension, due to reduced systolic arterial pressure, fibrosis, and sympathetic hyperactivity (Sales et al., 2015).

## 1.5. Stages in the development of renovascular hypertension

### 1.5.1. Renin angiotensin system (RAS) dependent phase

The renin angiotensin system-dependent phase is also known as the early phase of hypertension (Brown et al., 1976). Hypertension in this phase is mainly the result of excessive activation of the RAS. Diminished renal perfusion stimulates secretion of renin, which in turns leads to increased plasma Angiotensin II levels. The elevated blood pressure in this phase could be repaired and reversed by the inhibition or blocking of the renin angiotensin system, or by surgical repair of the renal artery stenosis (Pipinos et al., 1998).

### 1.5.2. Salt retention phase

In this transitional phase, hypertension is more likely to occur as a result of the sodium and water retention than an increase of Ang II. Activation of AT1R leads to several effects besides an increase in blood pressure, including induced salt retention and aldosterone

secretion (Robles et al., 2014) (Brewster & Perazella, 2004). Salt retention may suppress plasma and renal renin secretion resulting in raised systemic blood pressure, due to the lack of response to the blockage Ang II (Garovic et al., 2005)

### 1.5.3. Systemic renin angiotensin independent phase

Systemic renin angiotensin independent phase is also called the chronic or sustained late phase of renovascular hypertension. In contrast to the early phase, plasma renin level returns to normal, and in this case, hypertension cannot be reversed by surgical correction of the stenosis, and this condition is typically less responsive to RAS blockade (Pipinos et al., 1998).

### 1.6. Components of RAS cascade

### Renin

Activation of the RAS is initiated by the release of a protease enzyme, called renin, from the kidney, primarily synthesized by juxtaglomerular cells (Nguyen et al., 2002). Renin is also known as angiotensinogenase; it has a role in the cleavage of the glycoprotein angiotensinogen and in forming the inactive decapeptide angiotensin I (Ang I) (Nguyen et al., 2002). Renin secretion occurs in response to reduced arterial blood pressure, sodium level, and sympathetic nervous system activity (Ferrao et al., 2014).

### ACE/Angiotensin II/Angiotensin II receptor axis

The octapeptide Ang II is a highly active peptide of the RAS. It has an essential role in blood pressure regulation and body fluid homeostasis (Belova, 2000). The effect of Ang II is mediated by its binding to either the Ang II type 1 receptor (AT1R) or the Ang II type 2 receptor (AT2R) (Siragy & Carey, 2010). Binding of Ang II to AT1R leads to several physiological effects such as vasoconstriction, sodium retention, reduced glomerular

filtration rate, and may cause renal injury (Kobori et al., 2007). Ang II is considered an effective antinatriuretic hormone; it is responsible for the regulation of sodium reabsorption through the activation of renal Na+ /H+ exchanger 3 (NHE3) in the proximal tubule (Banday & Lokhandwala, 2011). It has been demonstrated that oxidative stress could increase AT1R signaling and over-stimulate NHE3, which may lead to down-regulated sodium excretion and ultimately hypertension (Banday & Lokhandwala, 2011).

## Angiotensin II Receptors subtypes

### - Angiotensin II type 1 receptor

The two main classes of Ang II receptors belong to the large family of seven trans-membrane receptors (Sparks et al., 2014). These two receptors activate by Ang II, but trigger counteractive mechanisms (Cresci et al., 2003). Binding of AT1R with Ang II modulates blood pressure, hormone secretion, and renal function (de Gasparo et al., 2000). In rats and mice, AT1R has two distinct subtypes, named $AT1_A$ and $AT1_B$ (de Gasparo et al., 2000); however, these subtypes have not been discovered in humans yet (Bergsma et al., 1992). $AT1_A$ and $AT1_B$ have similar binding properties, but differ in the genomic structure, distribution, and transcriptional regulation (de Gasparo et al., 2000). AT1R is expressed in different tissues; however in kidneys it is mainly located in glomerular mesangial cells, proximal tubular cells, and interstitial cells (de Gasparo et al., 2000). Most studies suggested that the predominate type of these receptors in the RAS is the type 1 receptor (Timmermans et al., 1993). AT1R has several functions, including vasoconstriction effects that lead to high blood pressure via Ang II activation, sodium balance, growth regulation, and developmental regulation (Oliverio & Coffman, 2000). The up-regulation of AT1R via oxidative stress can lead to excessive sodium transporter stimulation and decrease in sodium excretion, which contribute to an increase in blood

pressure (Banday & Lokhandwala, 2011). Several past studies are in agreement with the notion that a raise in blood pressure, via Ang II activation, is mainly mediated through AT1R (Ito et al., 1995; Crowley et al., 2004; Crowley et al., 2010; Balakumar & Jagadeesh, 2014). Accordingly, the use of AT1 blockers (ARBs) is recommended in the management of hypertension and for delaying the progression of CKD (Imaizumi et al., 2013; Balakumar & Jagadeesh, 2014).

- Angiotensin II type 2 receptor

AT2Rs antagonize the actions of AT1Rs (Jones et al., 2008). The low expression of AT2Rs relative to AT1Rs leads to the idea that AT2Rs may have a negligible biological impact (Padia & Carey, 2013). However, recent studies showed that AT2R activation might have a protective role in hypertensive patients via vasodilation, and prevention of sodium retention (Kemp et al., 2014; Danyel et al., 2013). Interestingly, expression and function of the renal AT2R depends on sex (Hilliard et al., 2012). Studies show that activation of the endogenous AT2R in the brain, specifically in females, may play a protective role in deoxycorticosterone acetate (DOCA) salt-induced hypertension (Dai et al., 2015). Thus, the use of AT2R could be considered an effective promising target in the treatment of fluid retention and hypertension.

**ACE2/Angiotensin (1-7)/mas receptor axis**

ACE2 plays a key role in the cleavage of one amino acid from Ang I and Ang II to form Ang (1-9) and the heptapeptide Ang (1-7), respectively (Batlle et al., 2012). The balance between ACE and ACE2 in the RAS is required to regulate Ang II levels. The formation of the heptapeptide Ang (1-7) occurs either directly from Ang I through neutral endopeptidase NEP and propyl endopeptidase (PEP) (Grobe et al., 2013), or from Ang II

by angiotensin-converting enzyme 2 (ACE2) and prolyl carboxypeptidase (PCP) (Eriksson et al., 2002; Donoghue et al., 2000; Odya et al., 1978). Ang (1-7) is found in the heart, kidney, blood vessels, and liver (Santos et al., 2013). It antagonizes the effects of Ang II by acting as a vasodilator when it binds to two receptors either the G-protein coupled receptor mas (Batlle et al., 2012) or AT2R (Santos et al., 2003). Activation of these receptors may lead to many biological processes including proliferation, fibrosis, hypertrophy, and thrombosis (Bian et al., 2013; Macedo et al., 2014). A study suggested that the counterbalancing effect of the ACE2-Ang (1-7)-mas axis is associated with reduced blood pressure in the 2K1C Goldblatt hypertension model (Rakusan et al., 2010). The protective role of Ang (1-7) in the renal and cardiovascular system is well documented. Moreover, the beneficial effects of Ang (1-7) have also been shown in several organs, such as cerebro-protection against ischemic stroke (Mecca et al., 2011), gastro-protection against gastric lesions (Brzozowski et al., 2012), and also protection against diabetic retinopathy (Verma et al., 2012). Therefore, the ACE2-Ang (1-7)-mas axis could be a new promising target for the development of potential therapies for different disorders.

**Angiotensin (1-7) forming enzymes**

**- Angiotensin-converting enzyme 2 (ACE2)**

ACE2 is a monocarboxypeptidase and the first human homolog of ACE (Donoghue et al., 2000). It removes the C-terminal amino acid from several substrates including Ang I to form Ang (1-9) and Ang II to give Ang (1-7) (Batlle et al., 2012). Originally, ACE2 was identified in the heart, testis, and kidney (Donoghue et al., 2000). However, ACE2 has also been observed in others organs, such as the lung, liver, pancreas, adipose, retina,

ovary, colon, small intestine, and rat brain (Bindom & Lazartigues, 2009). In the kidney, most recent studies have shown that ACE2 is predominantly expressed in the renal cortex (Santos et al., 2013), mainly in glomerular podocytes and in the cell wall of Bowman's capsule (Ye et al., 2006), while ACE by contrast, is expressed mostly in endothelial cells (Ye et al., 2006). Notably, there was no ACE2 in the plasma of *db/db* diabetic mice or in control animals (Chodavarapu et al., 2013). Recent clinical studies reported that treatment of patients with hypertension or with diabetic nephropathy with Olmesartan, one of the ARBs, (Abe et al., 2014) (Furuhashi et al., 2015) resulted in increased urinary ACE2, suggesting a potential renoprotective role of ACE2.

- Neutral endopeptidase neprilysin (NEP)

Neutral endopeptidase neprilysin (NEP, EC 3.4.24.11) belongs to the M13 family of zinc-containing metallopeptidases (Sexton et al., 2012). It is also known as CD10, enkephalinase, or common acute lymphoblastic leukemia CALLA (Sexton et al., 2012). The molecular weight of NEP is around 90-110 kDa, with a short cytoplasmic and a large extracellular domain (Zraika et al., 2007). Initially, NEP was discovered in the brush border membranes of the rabbit kidney as a cleavage peptide of the insulin B chain (Kerr & Kenny, 1974). NEP is expressed in different tissues such as lung, brain, fibroblasts, various epithelia, vascular cells, and neural synapses of the central nervous system (Kerr & Kenny, 1974; Li et al., 1995). Degradation of Ang I by NEP forms the vasodilator Ang (1-7), step advantageously used for vascular therapeutic applications (Chappell, 2007; Simoes e Silva AC et al., 2006). Apart from Ang I, NEP also degrades other biologically active peptides such as bradykinin, atrial natriuretic peptide (Cruden et al., 2004), B-type natriuretic peptide (Judge et al., 2014), and C-type natriuretic peptide (Thong et al., 2014).

Accumulating evidence suggests a role of NEP in Alzheimer's disease (Park et al., 2013). Indeed, NEP degrades amyloid β peptides (Aß); thus, NEP deficiency could be associated with the accumulation of Aß in cerebral regions, the main pathological characteristic of Alzheimer's disease (Yasojima et al., 2001; Iwata et al., 2001). Furthermore, NEP may have a therapeutic protective role in pancreatic beta cells via the degradation of islet amyloid polypeptide (IAP) (Guan et al., 2012). NEP has been proposed as a diagnostic marker of different renal neoplasms (Avery et al., 2000). Moreover, a previous study suggested that down regulation of NEP expression is an essential factor in the progression of prostate cancer (Papandreou et al., 1998). This was followed by a recent study highlighting the need for early biomarkers like NEP to classify different cancer cell phenotypes (Ho et al., 2013).

## 1.7. Role of A Disintegrin and Metalloprotease 17 (ADAM17) in renal ACE2 shedding

ADAM17 is a member of A Disintegrin and Metalloprotease family, also known as tumor necrosis factor α-converting enzyme (TACE) (White, 2003). ADAM17 is expressed in several tissues including brain, heart, kidney, and skeletal muscle and is involved in the ectodomain shedding of several transmembrane proteins (Black et al., 1997). The ectodomain shedding process is defined as the hydrolytic breakdown of transmembrane proteins resulting in the release of their extracellular domains (Mezyk et al., 2003). Ang II infusion activates ADAM17, which acts as a sheddase for transforming growth factor-alpha (TGF-α), suggesting a new therapeutic approach for ADAM17 inhibitor in the prevention of CKD (Lautrette et al., 2005). A recent study reported the role of ADAM17 in the release of NEP via exosomes from endothelial cells (Kuruppu et al., 2014). Our previous studies demonstrated the critical role of ADAM17 in the shedding of renal ACE2

in type 2 diabetic mice and the effect of using an insulin sensitizer, such as rosiglitazone, on the expression of renal ADAM17 and ACE2 proteins (Chodavarapu et al., 2013). Furthermore, Somineni *et al.* showed that daily exercise training attenuates renal ACE2 shedding through the reduction of renal ADAM17 protein level on *db/db* diabetic mice (Somineni et al., 2014). Emerging studies reported that ADAM17 expression and activity increase in several disorders such as akita and OVE26 mouse models of type 1 diabetic (Ford et al., 2013; Salem et al., 2014), heart failure, atherosclerosis, cancer, immune and neurological diseases (Mendoza & Lazartigues, 2015). Additionally, a recent report showed the increase of ADAM17 expression in the brain of deoxycorticosterone acetate (DOCA) salt induced-hypertension mice result in an increase of ACE2 shedding from the plasma membrane, which may contribute to the development of neurogenic hypertension (Mendoza & Lazartigues, 2015; Xia et al., 2013).

## 1.8. Management of renovascular hypertension

There are several options in the management of RVH. These involve pharmacological intervention, percutaneous intervention by transluminal angioplasty and stinting, and surgical intervention, which includes endarterectomy, aortorenal bypass, and extra-anatomic bypass (Bloch & Basile, 2007). Usually, medical therapy in combination with other interventions is considered more effective than using medical therapy alone (Garovic & Textor, 2005). Although several different antihypertensive drugs have been used to manage renovascular hypertension, ACE inhibitors and ARBs are, for now, considered the ultimate effective drugs (Safian & Textor, 2001).

### 1.8.2.1 ACE inhibitors

The affectivity of ACE inhibitors in the management of RVH is long recognized (Reams & Bauer, 1985; Schwietzer & Oelkers, 1982). It has been proposed as the most effective therapy when compared to other antihypertensive drugs available (Hackam et al., 2007). The use of ACE inhibitors is especially important in patients with atherosclerotic renal artery stenosis (Tullis et al., 1999) and in non-diabetic patients with kidney disease in order to prevent development of nephropathy (Tylicki et al., 2012). The renoprotective roles of these inhibitors have been demonstrated in several studies (Huang et al., 2014). Both ACE inhibitors and ARBs have similar efficiencies in the control of blood pressure and in renal protection; however a significant side effect of ACE inhibitors, when compared to ARBs, is the high possibility to develop dry cough and angioedema (Matchar et al., 2008).

### 1.8.2.2 Angiotensin II receptor blockers (ARBs)

Apart from the use of ARBs to control blood pressure, ARBs have also been used to treat heart failure, coronary heart disease, and diabetic nephropathy (Schmieder et al., 2011). Unlike ACE inhibitors, several studies discovered that ARBs are less likely to cause dry cough and angioedema, due to reduced breakdown of bradykinin (Mallat, 2012; Yusuf et al., 2008). Moreover, clinical data reported that ARBs exhibit little or no effect in hypertensive patients with hyperlipidaemia (Nishida et al., 2011). In type 2 diabetic patients who have microalbuminuria, there is a similar beneficial effect of ACE inhibitors and ARBs. However, clinical trials recommend the use of ARBs in diabetic patients with proteinuria, as it is more effective in delaying the progression of nephropathy (Mallat, 2012).

### 1.9. Chronic kidney disease: a risk factor for renovascular disease

The increase in prevalence of CKD in the United States is associated with the increasing prevalence of diabetes and hypertension, which may also lead to several complications such as cardiovascular disease and ESRD (Coresh et al., 2007). CKD is defined as the presence of any abnormality in the renal structure or in the renal function for more than 3 months, with some associated effects on health (Stevens & Levin, 2013). The National Kidney Foundation Guidelines classified CKD according to the glomerular filtration rate or kidney damage indicated by presence of albuminuria. Reduced GFR (<60 mL/min per 1.73m$^2$) is generally the best indicator of CKD. However, in stage 1 and 2 of CKD, GFR could be normal, and a diagnosis is not made. Therefore, recent guidelines recommend the use of albuminuria in addition to GFR to have an accurate assessment of CKD (Stevens & Levin, 2013). Moreover, GFR categories have been updated with new subdivision in the G3 stage with level of 30 to 59 mL/min per 1.73 m$^2$ as G3a for 45 to 59 mL/min per 1.73 m$^2$ and G3b for 30 to 44 mL/min per 1.73 m$^2$ (Stevens & Levin, 2013). Emerging studies showed that development of CKD is widely associated with activation of RAS system (Remuzzi et al., 2005).

### 1.10. Chronic kidney disease biomarkers

### 1.10.1. Albuminuria

Albuminuria is an indicator of renal dysfunction and is independently associated with renal and cardiovascular events (Stephen et al., 2014). Urinary proteins originate mainly from glomerular filtration of plasma proteins, such as albumin, while non-plasma proteins are produced by renal tubular epithelium (Stephen et al., 2014). Normally, albumin, which has a low molecular weight, is filtered by the glomerulus and most of it nearly reabsorbed by the renal proximal tubules (Barratt & Topham, 2007). The normal daily range of

albumin excretion is from 5 to 10 mg, and the urine albumin: creatinine ratio range from 0 to 29 mg albumin/g creatinine (Toto, 2004). When proteinuria endures, albumin is the most common protein found in urine. Microalbuminuria is defined when values range between 30–299 mg of albumin/g of creatinine, whereas the term macroalbuminuria is reserved when values ≥300 mg albumin/g creatinine are demonstrated (Toto, 2004; Stevens & Levin, 2013). However, recently the term micro and macro have no longer been used due to the continuous occurrence of albuminuria (American Diabetes Association, 2015). Our previous studies suggested a positive correlation of urinary ACE2 with albuminuria in both types of diabetes, type 1 diabetic Akita mice (Salem et al., 2014) and type 2 diabetic *db/db* mice (Chodavarapu et al., 2013; Somineni et al., 2014), a concept that could place ACE2 as a more sensitive early biomarker for CKD than albuminuria.

### 1.10.2. Estimated glomerular filtration rate (eGFR)

Estimation of glomerular filtration rate is considered the best indicator of renal function (Stevens & Levey, 2009). Clinically, estimated GFR (eGFR) usually depends on the endogenous serum creatinine marker more than urine collection (exogenous markers) (Rule et al., 2013). In addition, the eGFR equation includes other factors beside serum creatinine such as age, race, sex, and body weight (Stevens et al., 2006). Several studies have shown that eGFR based on serum creatinine is more related to CKD than eGFR based on cystatin C (Rule et al., 2013). However, recent studies reported that combining serum creatinine with cystatin C would be more effective and accurate in the assessment of renal function in CKD (Fan et al., 2014; Wasung et al., 2015). The MDRD (Modification of Diet in Renal Disease) equation is the most commonly used equation used in clinical laboratories to assess CKD (Miller, 2008). But recently, researchers have

developed a new equation known as the CKD-EPI (chronic kidney disease epidemiology collaboration) to give a more accurate assessment of GFR, especially in conditions with high eGFR levels (Levey et al., 2009).

### 1.10.3 The intrarenal RAS components as urinary biomarkers

Studies have suggested that increased levels of urinary angiotensinogen (UAGT) in CKD patients could be used as a reliable marker of CKD (Mills et al., 2012). Moreover, UAGT level is also increased in hypertensive subjects (Kobori et al., 2009). These data suggest that UAGT may provide an accurate reflection of intrarenal RAS and may be considered an early indicator for several disorders including CKD, diabetic nephropathy, and renovascular hypertension (Kobori et al., 2009) (Kamiyama et al., 2012). However, Ang II could not be considered as an intrarenal RAS biomarker due to its unstable status (Mills et al., 2012). Additionally, a study was done by Casarini and her colleagues that established the role of urinary ACE as an indicator of hypertension in order to avoid the development of RVH and/or cardiovascular disease (Maluf-Meiken et al., 2012).

### 1.10.4 Tubular injury biomarkers

Elevated urinary kidney injury molecule-1 (KIM-1) was found in the injured proximal cells in animal models and humans (Zhang et al., 2007). Urinary KIM-1 levels have been proposed as a potential indicator of CKD progression. It is considered an optimal marker, for at least three reasons: its ectodomain is rapidly chopped and detectable in the urine, it is specific to renal tubular damaged cells and not detectable in normal cells, and it shows sensitivity in the urinary quantification of kidney injury assessment and for monitoring therapeutic effects (Huo et al., 2010). A study in 2014 examined and confirmed the

association between kidney tubular damage and cardiovascular disease progression and high levels of urinary KIM-1 (Carlsson et al., 2014).

Other endogenous markers currently used in clinical laboratory tests to assess GFR include serum creatinine and cystatin C (Herget-Rosenthal et al., 2007). Cystatin C is also considered a tubular injury biomarker, mainly for early kidney dysfunction. In fact, Cyctatin C in combination with serum creatinine provides a more accurate assessment of GFR (Wasung et al., 2015).

Neutrophil gelatinase-associated Lipocalin (NGAL) is a member of the Lipocalin family, mainly found in neutrophils and tubular epithelial cells (Cowland et al., 2003). NGAL increases in response to inflammatory conditions and ischemia (Eirin et al., 2012). Urinary and plasma NGAL are currently considered reliable markers of acute kidney injury and chronic RVH (Eirin et al., 2012).

Studies of RAS activation and its components in hypertensive conditions are limited. The present thesis will examine the expression of renal and urinary ACE2 and NEP and their potential renoprotective effect in the 2K1C renovascular hypertensive mouse model. We will investigate the role of AT1$_A$R in general functional parameters, including renal function, RAS components and its association with CKD and RVH.

## 2. HYPOTHESIS AND SPECIFIC AIMS

### 2.1. Hypothesis

To test the hypothesis that: 1) increased blood pressure and renal ADAM 17 protein expression in the 2K1C mouse model of renovascular hypertension increase shedding of renal ACE2 and NEP which may contribute to the progression of chronic kidney disease in this model and 2) hypertension and shedding of renal ACE2 and NEP is mediated by $AT1_AR$

### 2.2. Specific aims

1. To test the hypothesis that hypertension in the 2K1C model of renovascular hypertension is mediated via $AT1_AR$.

2. To test the hypothesis that deleting $AT1_AR$ in the 2K1C model of renovascular hypertension will attenuate the increased urinary albuminuria excretion.

3. To test the hypothesis that there is increased shedding of renal ACE2 into the urine in 2K1C mice, which could be mediated through $AT1_AR$, and to investigate whether this is associated with increased renal ADAM17.

4. To investigate whether renal and urinary NEP protein expression are altered in 2K1C mice.

## 3. MATERIAL AND METHODS
### 3.1. Animal model

Adult male $AT1_A-/-$ (AT1 KO) and their wild type control $AT1_A+/+$ (WT) mice were used. The mice were generated from a breeding colony maintained at Wright State University. The breeding stock mice were provided by Dr. Thomas Coffman (Duke University, Durham, NC, USA) (Ito et al., 1995). Mice were housed individually in plastic cages with ad libitum access to food and water and were maintained at room temperature (22°C) with 12:12 h light: dark cycles. Mice were randomly divided into four groups: WT (sham), WT (2K1C), AT1 KO (sham), and AT1 KO (2K1C).

Every alternate day, body weight, food intake, water intake, urine output, and blood pressure of the mice were monitored. The Wright State University Animal Care and Use Committee approved all the experimental protocols.

### 3.2. Telemetry transmitter implantation

Telemetric catheters devices (model TA11PA-C20) were purchased from Data Sciences (Data Sciences International, St. Paul, MN). In a closed chamber, mice were initially anesthetized with 2.5% isoflurane and 1 L/min oxygen and then maintained with 2% isoflurane and 0.3-0.5 L/min oxygen on a constantly warm pad using a nose cone. Mice were placed on their backs using tape to secure their forelimbs. For surgical preparation, the anterior neck was shaved and disinfected with Betadine solution and 70% ethanol. A small incision (10 mm) was made vertically through the neck, and carefully, a sterile telemetry catheter was inserted into the carotid artery and tied in place using a 5.0 gauge silk suture. A subcutaneous sack was made on the animal's flank to place the transmitter device inside the body, and then the skin was closed with 5.0 sutures and disinfected with

Betadine solution. A dose of 0.1 mg/kg of Carprofen was given subcutaneously to each animal immediately after surgery and on the next day for pain reduction to provide 48 hours of analgesia. All mice were left to recover and rest for 7 days before recording the blood pressure. Measurements were taken for 24 hours as baseline prior to the 2K1C renal clipping surgery. At the end of the study period, mice were sacrificed using carbon dioxide.

### 3.3. 2K1C surgical procedure

Mice were anesthetized with isoflurane (induction 2.5% isoflurane, maintenance 2% isoflurane) and kept on a warm pad to avoid hypothermia. U-shaped sterile stainless steels clips (0.12-mm, Exidel SA, Switzerland) were used to clip the renal artery. The clip was placed on the renal artery of the left kidney after it was isolated through a flank incision by using forceps. The kidney was then returned into the retroperitoneal cavity and the abdominal wall layers were sutured. The same surgical procedure was done for sham animals to dissect the left renal artery with the exception of clipping, in order to use them as controls. Carprofen (0.1 mg/kg) was given subcutaneously to mice immediately after surgery to reduce pain and also after 24 hours to provide 48 hours of analgesia. Postoperatively daily assessment of general health, suture site, body weight, water, and food consumption was performed.

### 3.4. Radiotelemetry measurements

Arterial blood pressure, heart rate, and locomotor activity were continuously monitored using radiotelemetry (500 Hz) before (baseline period) and up to two weeks after the 2K1C renal clipping surgery. The standard Ponemah Analysis Modules system (Data Sciences International, St. Paul, MN) was used for data analysis.

### 3.5. Twenty-four hour urine collection

Mice were housed individually in metabolic cages for 24-hour urine collection under a 12:12 hour light: dark cycle, provided with water and standard rodent chow. Ten microliters of protease inhibitor (Roche Diagnostics, IN, USA) containing 2.5 mM/L PMSF was added twice to the urine samples through the 24-hour period to prevent protein degradation. The samples were then centrifuged at 3,000 x g, 4° C for 5 min. The supernatants were aliquoted and stored at -80°C for later use.

### 3.6. Perfusion and tissue collection

Mice were anesthetized with 0.13 mg/g Euthasol intraperitoneally. Once adequate anesthesia was assured by checking the reflex and the mice showed no response to the tail/toe pinches, a thoracic midline skin incision was made to expose the abdomen and the thorax cavity completely. A small incision was made in the atrium and a 25-gauge needle was inserted into the left ventricle. The perfusion started by pumping the perfusate buffer (1X PBS, 60-70 ml/mouse) through the needle into the animal at a moderate flow. Once blood was cleared and the liver became pale, the perfusate buffer was switched to the fixative fluid (4% paraformaldehyde, 50-60 ml/mouse) until the mouse limbs became stiff. Organs then were collected in paraformaldehyde and kept at 4°C for later use.

### 3.7. Urinary creatinine assay

Urinary Creatinine levels are commonly used as an index for kidney function, due to its stability and excretion rate in a normal individual. Urinary creatinine protein was measured using a Creatinine ELISA kit purchased from Quidel Corporation BoneVue (San Diego, CA). The kit is based on the principle of Jaffe reaction under alkaline conditions. Creatinine reacts directly with picric ions to produce a color complex solution.

Standards and urine samples were diluted with distilled water in the ratio (1:40), and 50 µl of samples and standards were added into the 96 wells plate followed by addition of 150 µl of color reagent (7 ml picric acid + 1 ml 1N NaOH). The plate was incubated at room temperature for 30 minutes before taking the readings. Measurements were determined at 490 *nm* using a Fusion Packard plate reader.

### 3.8. Urinary albumin assay

Albuminuria is the current indicator for the assessment of kidney damage in CKD. Mouse Albumin ELISA Quantitation kit (Bethyl Laboratories, Montgomery, TX, USA) was used to determine the excretion amount of urinary albumin protein. Initially, the 96-wells plate was coated with 100 µl of goat anti-mouse Albumin antibody diluted in 10 ml carbonate-bicarbonate buffer for an hour at room temperature. After aspiration of the previous solution, the plate was washed 4 times with 250 µl washing buffer (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20). It was then blocked by adding 200 µl of blocking buffer (Tris buffered saline + 1% BSA) overnight at 4°C.

After overnight incubation, standards were prepared according to the kit's protocol and diluted with sample/conjugate buffer. Also, 24-hour urine samples were diluted in the ratio 1:1000 with sample/conjugate buffer. The plate was washed as previously described before adding 100 µl of standards and urine samples, and incubated at room temperature for an hour. The plate was washed once again before adding the secondary antibody. HRP-conjugated detection secondary antibody was diluted in the ratio 1:35000 with sample/conjugate buffer and a 100 µl were added to each well for one-hour incubation at room temperature. The enzyme substrate TMB Peroxide was prepared by mixing solution A with solution B in 1:1 ratio. It was then washed one more time and 100 µl of that

mixture was added to each well and kept in the dark at RT for 15 minutes. In order to stop the reaction, 100 µl of 2N $H_2SO_4$ was added into the wells and the final absorbance was determined at 450 nm using a Fusion Packard plate reader.

### 3.9. Renal and urinary NEP ELISA assay

Mouse NEP Duoset kit was purchased from R&D systems (Cat #DY1126, Minneapolis, MN, USA). It was used to quantify NEP in 24-hour urine and kidney lysate samples. Kidney tissues were homogenized on ice in phosphate buffered saline (PBS) in the presence of protease inhibitor (Complete lysis M, Roche diagnostics, Indianapolis, IN, USA) and 2.5 mmol/L PMSF. Homogenate samples were then centrifuged at 10,000 x g for 10 min at 4°C to remove cellular debris. The supernatants were collected and the total protein content was measured for each sample using a BioRad reagent (Hercules, CA, USA) and BSA as a standard. The assay started with the 96-wells plate coated with a 100 µl of goat anti-mouse NEP capture antibody diluted in PBS and incubated overnight at room temperature. After incubation, PBS with 0.05% Tween 20 was used to wash the plate 3 times by adding 300 µl in each well. It was then blocked for an hour at RT using reagent diluent buffer (1% BSA in PBS). Standards and sample dilutions were prepared according to the kit's protocol; diluted in reagent diluent buffer and a 100-µl aliquot was added to each well and incubated for 2 hours at RT. The plate was washed 3 times as previously described and incubated with biotinylated goat anti-mouse NEP detection antibody (100 µl) for 2 hours at RT. Working solutions of Streptavidin-HRP (streptavidin conjugated to horseradish-peroxidase) were added in the dark as stated in the kit's protocol and incubated for 20 minutes at RT. The plate was then washed 3 times before the substrate solution TMB (1:1 mixture of color reagent A ($H_2O_2$) + color reagent B

Tetramethylbenzidine) was added and incubated in the dark for 20 minutes at RT. Finally, the reaction was stopped using 2N $H_2SO_4$ and final absorbance was read at 450 nm in Fusion Packard plate reader.

### 3.10. Renal and urinary ADAM17 ELISA assay

The objective of this technique is to quantify ADAM17 in kidney lysate and 24-hour urine samples. The Human ADAM17 Duoset kit was purchased from R&D systems (Cat #DY930, Minneapolis, MN, USA). Kidney tissues were homogenized on ice in phosphate buffered saline (PBS) containing protease inhibitor and PMSF as described above. Total protein content was measured for each sample using BSA as a standard and BioRad reagent. The mouse anti-human ADAM 17 diluted Capture antibody was used to coat the plate overnight at RT. After incubation, the plate was washed 3 times with PBS +0.05% Tween 20, and the blocking step was performed for an hour at RT using reagent diluent buffer (1% BSA in PBS). Standards and samples dilutions were prepared as stated in the kit's protocol; 100 μl were added and incubated for 2 hours at RT. After incubation, the plate was washed as described above and incubated with 100 μl in each well with biotinylated goat anti-human ADAM 17 detection antibody for 2 hours at RT. The working solution of Streptavidin-HRP and substrate solution TMB (1:1 solution A + solution B) were added in the dark according to the kit's protocol. After 15 minutes of adding the substrate solution TMB, the reaction was stopped by 2N $H_2SO_4$ and absorbance was read twice at 540 nm and 450 nm with a Fusion Packard plate reader, and final absorbance was measured by subtracting the two readings.

### 3.11. Western blot analysis

This immunoblotting technique was used to detect the expression of specific proteins in a

24-hour urine and kidney lysate samples. Homogenization of kidney tissues was performed as previously described (see section 3.9). Loading buffer (Laemmli Sample Buffer + 5% β mercaptoethanol, Bio-Rad, Hercules, CA) was used to prepare samples in the ratio 1:1, followed by boiling at 96$^o$C for 5 minutes. According to the total protein content, thirty micrograms of kidney lysates were loaded onto gels, while three micrograms of creatinine of urine samples were added to each well of a 10% sodium dodecyl-sulfate polyacrylamide gel (SDS-PAGE gel), followed by electrophoresis for one hour to separate the proteins. Using a Bio-Rad transfer apparatus (Hercules, CA), the proteins on the gel were transferred to a 0.2-μm polyvinylidene fluoride membrane (Millipore, MA, USA), which had been activated by methanol for 5 min. The membrane was blocked for an hour using 10% non-fat milk in 10 mM Tris buffer saline with Tween 20 (TBS-T) at RT to avoid non-specific binding. Primary antibodies dilutions were used as follows: goat anti-ACE2 (1:1000, R&D Systems, MN, USA), goat anti-ACE (1:250, Santa Cruz, CA, USA), rabbit anti-ADAM17 (1:500, Enzo Life Sciences, NY, USA), goat anti-Albumin (1:500, Santa Cruz, CA, USA), and goat anti-NEP (1:500, R&D system, MN, USA). The secondary antibodies were used accordingly as follows: HRP-conjugated donkey anti-goat (1:2000 R&D Systems, USA) or donkey anti-rabbit (1:20000, Jackson ImmunoResearch, USA). Blots were detected using super-signal chemiluminescent substrate (Thermo Scientific, IL, USA) and visualized using ChemiDoc imaging system (BioRad, Hercules, CA, USA). The intensity protein bands were quantified using Image lab 4.0 software (BioRad, USA).

### 3.12. Urinary and plasma ACE2 activity

The fluorogenic peptide substrate 7-Mca-APK (Dnp) is a specific substrate for ACE2

(Biomol International, NY, USA). The principle of the assay is based on the active protease ACE2 when it cleaves the substrate at a peptide bond between the fluorescence Mca and the quencher Dnp to produce fluorescence. After cleavage, the fluorescence signal is quantified by using a Fusion Packard plate reader set to read at excitation ($\lambda_{ex}$): 328 nm and emission ($\lambda_{em}$): 393 nm. To avoid interference with the endogenous ACE, an ACE inhibitor was used (10µM lisinopril) in the measurement of ACE2 activity. Two micrograms of creatinine from urine samples were added directly to the 96 wells plate, followed by incubation with 100 µl of ACE2 reaction buffer (50 mM Tris, 5 mM ZnCl, 150 mM NaCl$_2$ and 10 µM lisinopril) and Mca-APK (Dnp) (Enzo Life Sciences, Farmingdale, NY). Readings were taken at 0, 1, 2, 3, 4, and 6 hours. Results of urine samples were normalized according to urinary creatinine and expressed as nmol/h/mg creatinine.

### 3.13. Immunohistochemistry

The purpose of immunofluorescence experiments is to determine the localization and co-localization of proteins of interest using specific antibodies. Kidney tissues were collected from mice after the perfusion process as mention above and kept in paraformaldehyde at 4$^o$C. To obtain paraffin-embedded kidney sections and to stain them with periodic acid Schiff (PAS), kidney tissues were sent to AML laboratories (Baltimore, MD, USA). The procedure started by deparaffinizing paraffin sections with xylene for 5 min, followed by rehydrating using graded concentrations of ethanol (100%, 95%, 70%, 50%, and 30% for 5 min each). The slides were rinsed in distilled H$_2$O (dipping slides 4-5 times) and then in PBS for 10 min. In a water bath (95-99$^o$C), slides were placed into a plastic container filled with 10mM sodium citrate buffer for 30 min. After that, the slides were transferred

to another container filled with 1X PBS for washing twice (5 min for each). Slides were then blocked using 3% normal donkey serum and diluted with 1X PBS containing 0.1% Triton-X at 4˚C for an hour. Primary antibodies were used as follows: goat anti-NEP (1:500, R&D system, MN, USA) and rabbit anti-ADAM17 (1:100, Abcam system, MA, USA) diluted in 3% normal donkey serum, and incubated overnight at 4°C.

The next day, slides were washed once again 3 times with 1X PBS before incubation with the following secondary antibodies: CY3 conjugated donkey anti-goat (1:500, Jackson Immunoresearch, PA, USA) and FITC conjugated donkey anti-rabbit (1:100, Jackson Immuonoresearch, PA, USA) for 2 hours at 4˚C. Slides were allowed to air-dry and then were mounted using vectashield-mounting medium (Vector, Burlingame, CA, USA). Images were taken using a conventional fluorescence microscope (Optronics, Goleta, CA). Images were quantified using MetaMorph software (Molecular Devices, CA, USA).

### 3.14. Statistical analysis

Graph pad prism 5.01 and Image Lab software were used to analyze the data. All the statistics are represented as means ± SEM. The comparison of differences between the groups was calculated using the Student's unpaired two-tailed t-test. One-way ANOVA was applied for more than two groups, while two-way ANOVA used for multiple comparison among two or more groups, followed by Bonferroni's multiple comparison test. Statistically significance of the data was determined as $p<0.05$.

**Table 2: Effect of renal artery clip placement on general physiological parameters**

| Parameters | WT (Sham) | WT (2K1C) | AT1KO (Sham) | AT1KO (2K1C) |
|---|---|---|---|---|
| Age (weeks) | 12-19 | 12-19 | 12-19 | 12-19 |
| Time after surgery (weeks) | 2 | 2 | 2 | 2 |
| Body Weight (g) | 29.6 ± 0.5 | 29.2 ± 0.4 | 31.5 ± 0.3 | 28.6 ± 0.2 |
| Food intake (g/day) | 4.7 ± 0.1 | 4.6 ± 0.1 | 5.3 ± 0.2 | 5.2 ± 0.3 |
| Water intake (ml/day) | 6.6 ± 0.4 | 8.8 ± 1.2* | 9.9 ± 0.7 | 10.9 ± 0.6 |
| Urine volume (ml/day) | 1.2 ± 0.1 | 4.1 ± 1.5* | 4.5 ± 0.4 | 4.5 ± 0.5 |
| Creatinine concentration (mg/ml) | 0.4±0.03 | 0.1±0.03* | 0.2±0.02 | 0.1±0.02 |

Values represented as mean ±SEM. $^*p<0.001$ vs. age-matched WT sham mice were statistically significant.

**(A)**

**(B)**

**(C)**

**Figure 1: Effect of 2K1C on body weight**

Body weight measurements in (A) WT sham (white bar) and WT 2K1C (pink bar) (B) AT1 KO sham (black bar) and AT1 KO 2K1C (blue bar) (C) WT (white bar) and AT1KO (black bar). Repeated measurements two-way ANOVA using a Bonferroni's posthoc test showed no statistical difference in body weights of WT and AT1 KO mice at the baseline (0) and after sham or 2K1C surgical procedures during the two weeks of the study period. Data are represented as mean ± SEM, (n=5-8).

**(A)**



**(B)**
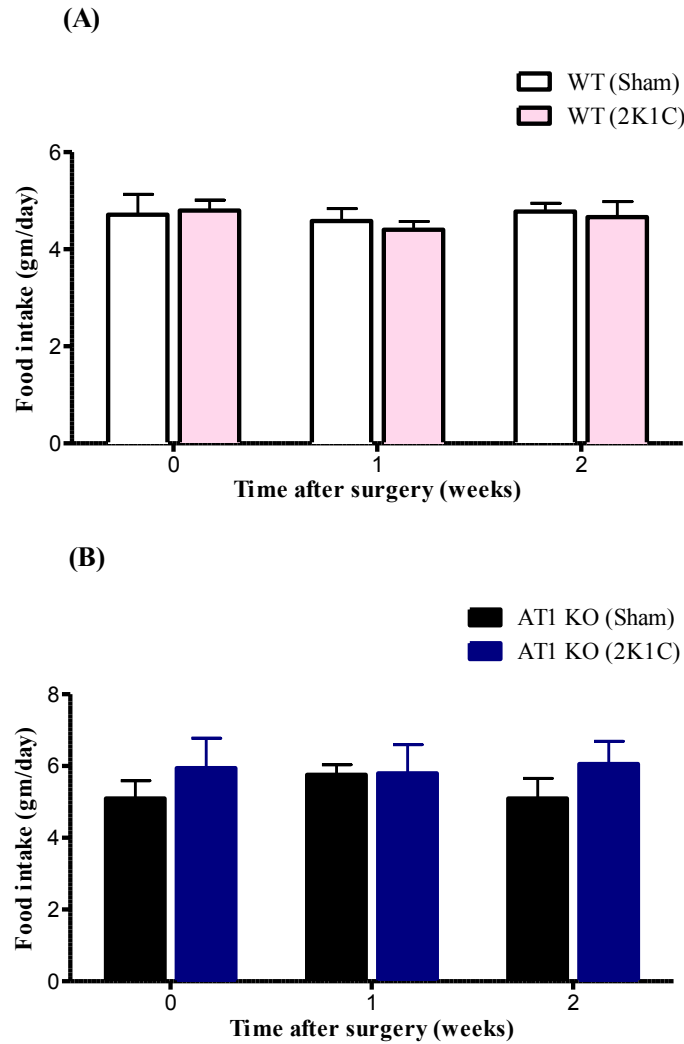


**Figure 2: Effect of 2K1C on food intake**

Food intake measurements in (A) WT sham (white bar) and WT 2K1C (pink bar) (B) AT1 KO sham (black bar) and AT1 KO 2K1C (blue bar). Repeated measurements two-way ANOVA using a Bonferroni's posthoc test showed that there was no statistical difference in food intake before (0) and after sham or 2K1C surgical procedures during the two weeks of the study period. Data are represented as mean ± SEM, (n=5-8).
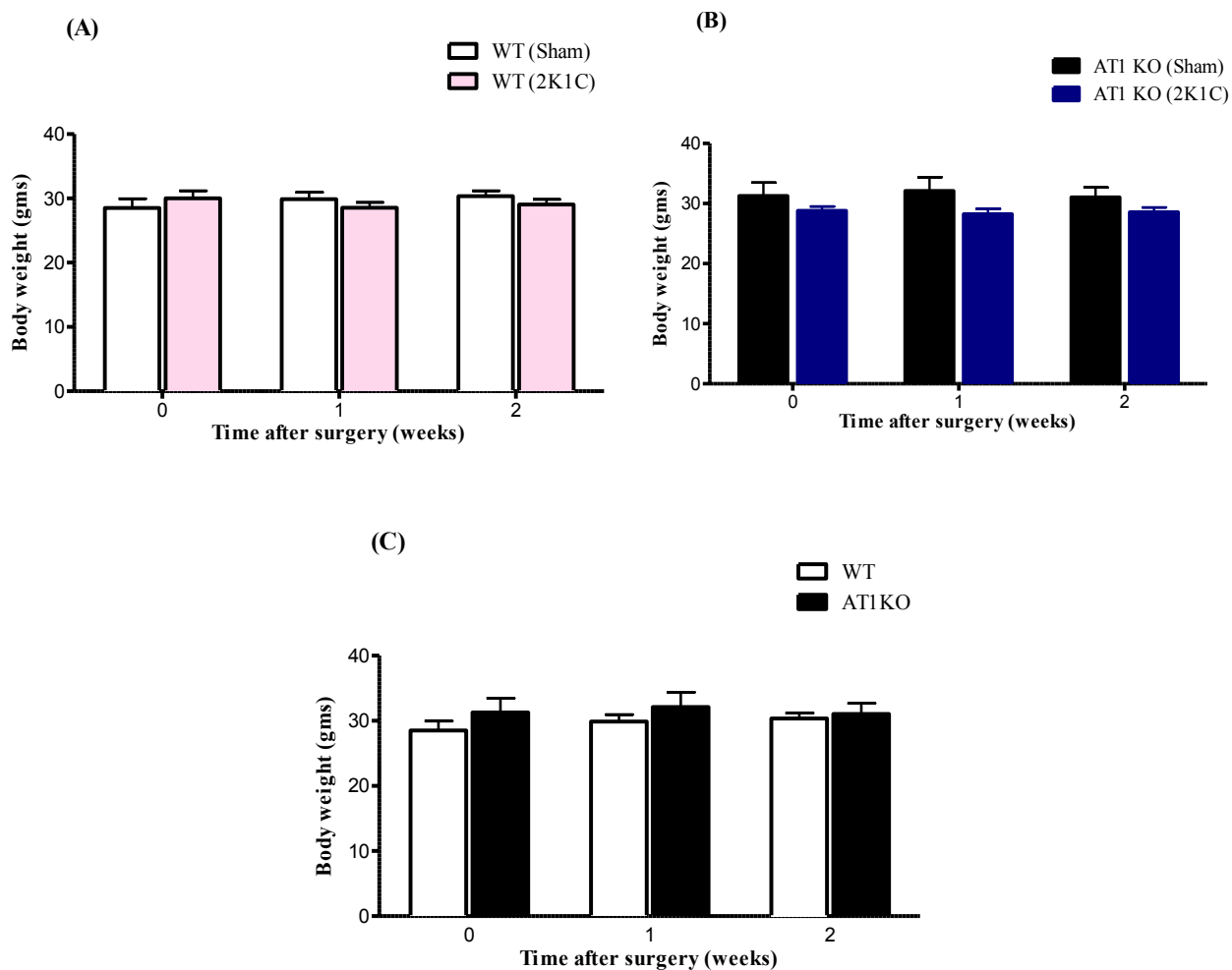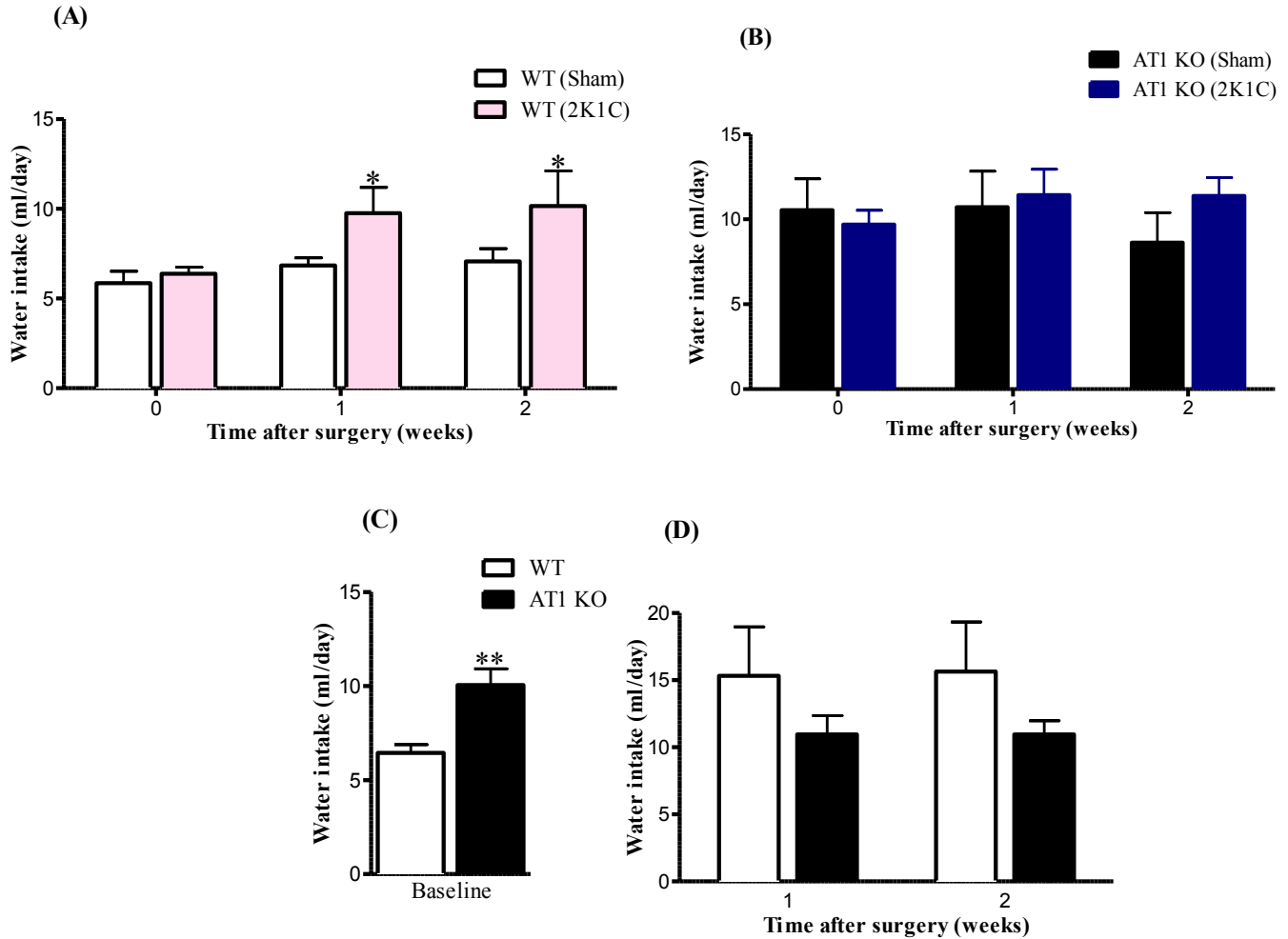
**Figure 3: Effect of 2K1C on water intake**

Water intake measurements in (A) WT sham (white bar) and WT 2K1C (pink bar) (B) AT1 KO sham (black bar) and AT1 KO 2K1C (blue bar). Repeated measurements two-way ANOVA using a Bonferroni's posthoc test showed that WT mice have a significant increase in water intake after 2K1C during the two weeks of the study. As well there was an increase in AT1 KO mice after 2K1C but was not significant. (C) At the baseline, Water intake was significantly increased in AT1 KO mice ([**]$p<0.0001$) compared to WT mice, while (D) it was decreased in AT1 KO after 2K1C compared to WT but not significant. Data are represented as mean ± SEM, (n=5-8).
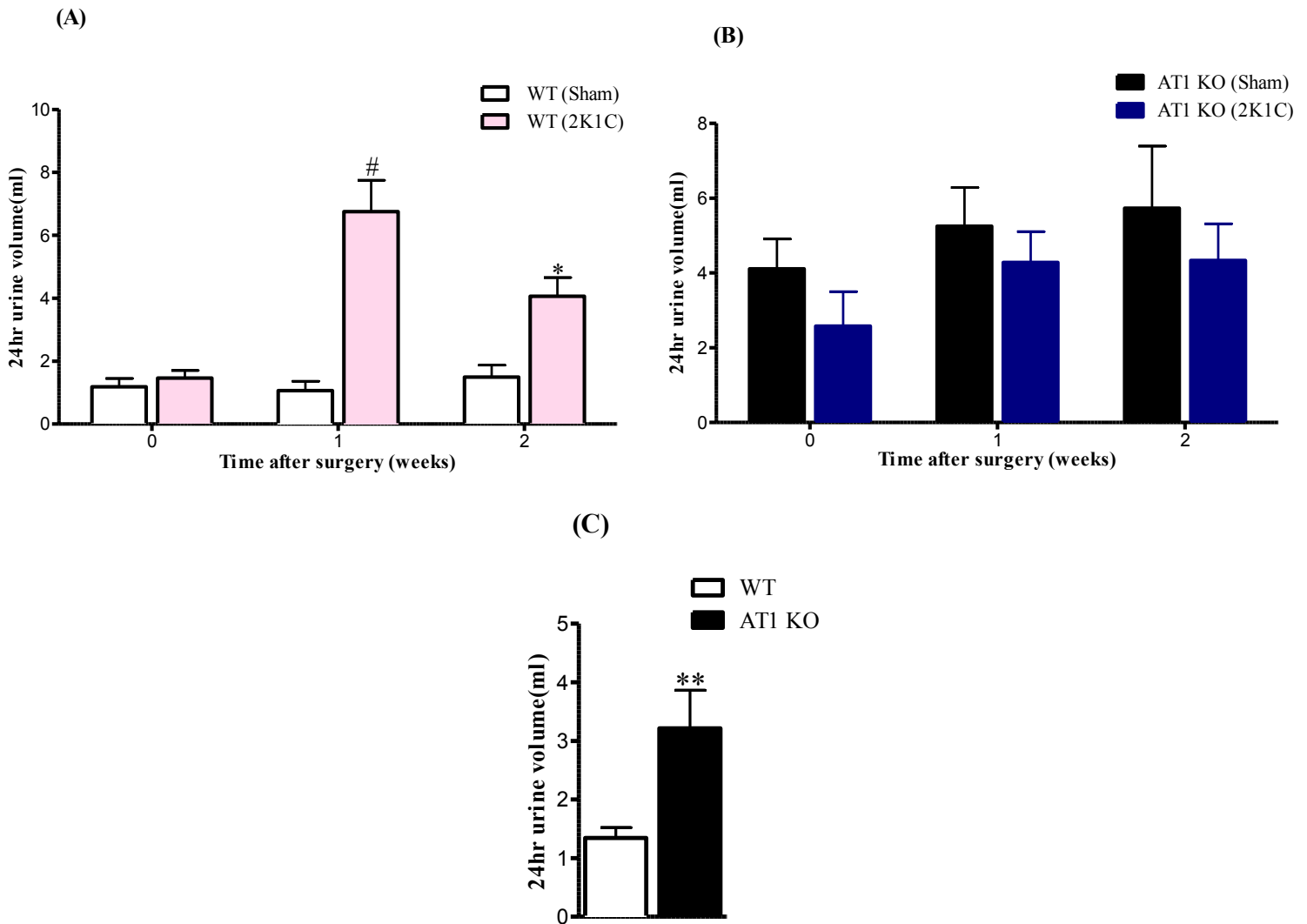
**Figure 4: Effect of 2K1C on urine output**

24-hour urine output measurements in (A) WT sham (white bar) and WT 2K1C (pink bar) Repeated measurements two-way ANOVA using a Bonferroni's posthoc test showed that daily urine volume was significantly increased in WT mice after 2K1C applied at the first week by $^{\#}p<0.0001$, and in the second week by $^{*}p<0.05$ vs. time matched WT sham mice. (B) AT1 KO sham (black bar) and AT1 KO 2K1C (blue bar). Repeated measurements two-way ANOVA using a Bonferroni's posthoc test showed that daily urine volume was increased but not significant in AT1 KO mice after 2K1C applied during the two weeks of the study period. (C) At the baseline, the 24-hour urine output was significantly increased in AT1 KO mice vs. time matched WT mice ($^{**}p<0.001$). Data are represented as mean ± SEM, (n=5-8).
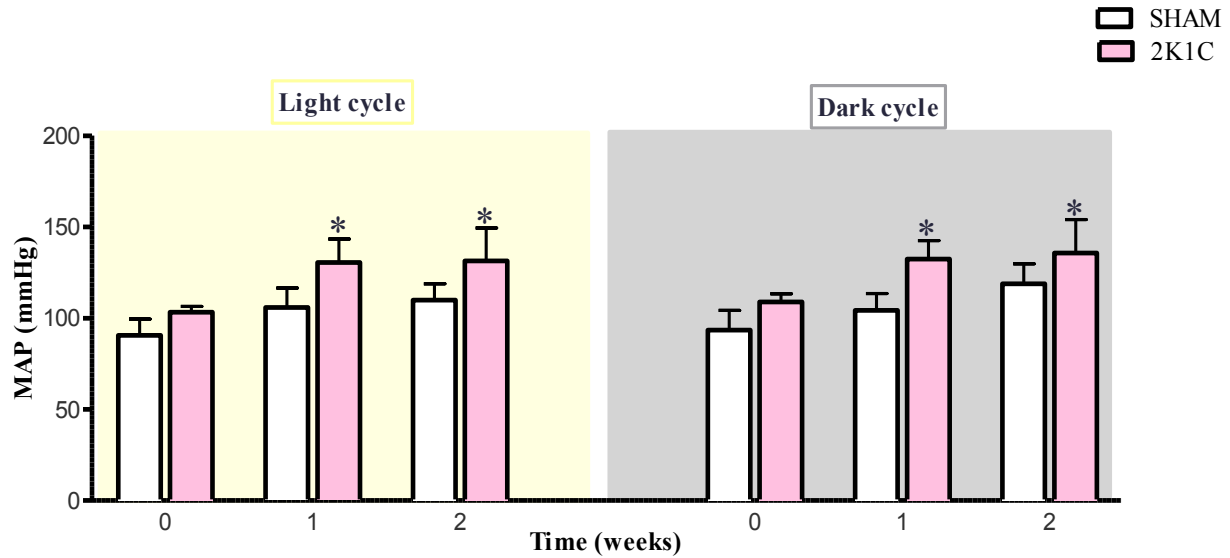
**Figure 5: Effect of 2K1C on mean arterial blood pressure in WT mice**

Mean arterial blood pressure (MAP) measurements in WT sham (white bar) and WT 2K1C (pink bar). Repeated measurements two-way ANOVA using a Bonferroni's posthoc test showed a significant increase in WT 2K1C mice when compared to baseline (0) and WT sham mice during the two weeks of the study ($^*p<0.05$). Data are represented as mean ± SEM, (n=5-8).

**(A)**



**(B)**
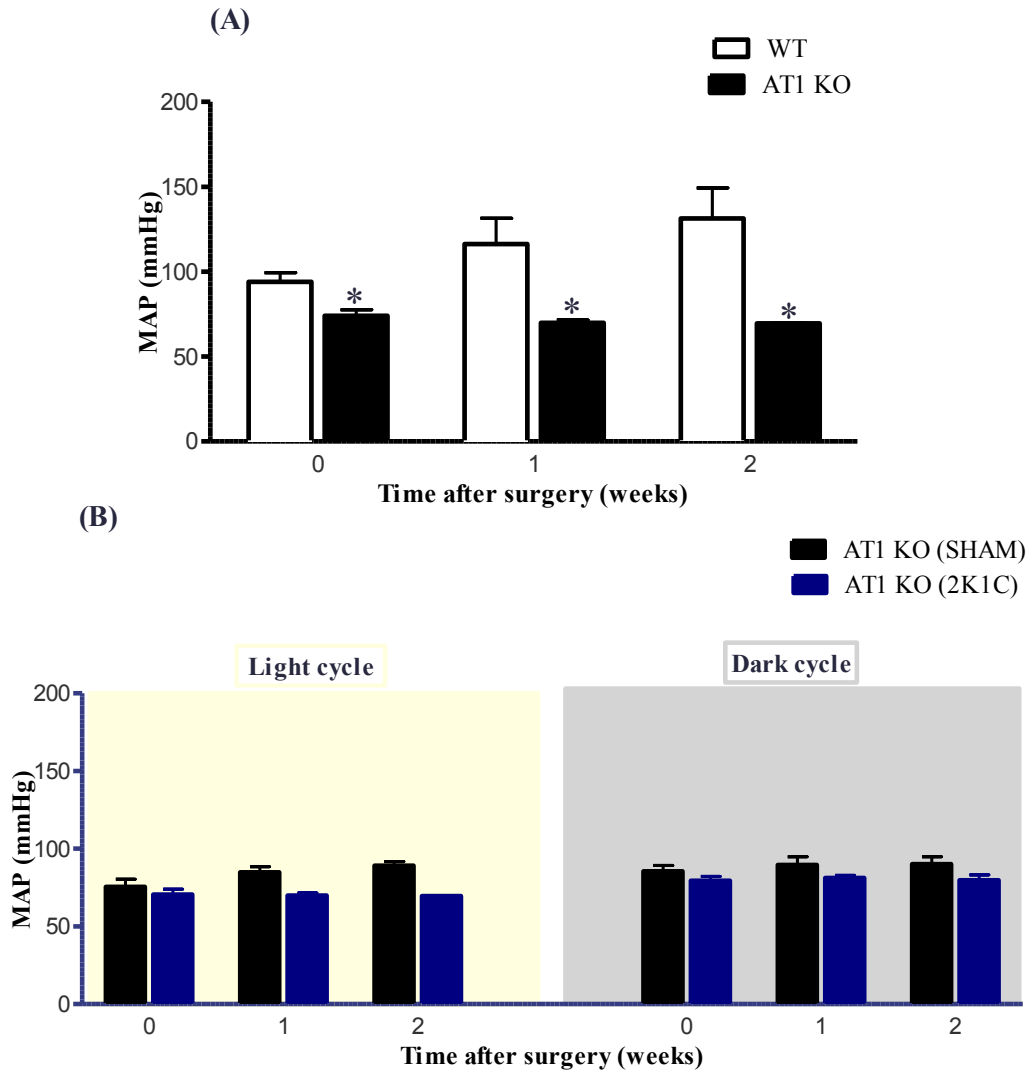


**Figure 6: Effect of 2K1C on mean arterial blood pressure in AT1 KO mice**

(A) MAP measurements in WT (white bar) and AT1 KO mice (black bar). One-way ANOVA showed a significant decrease in blood pressure of AT1 KO mice compared to WT mice before (0) and after 2K1C applied ($^*p<0.05$). (B) MAP measurements in AT1 KO sham and AT1 KO 2K1C mice. Repeated measurements two-way ANOVA using a Bonferroni's posthoc test showed no change in the blood pressure level of AT1 KO mice after 2K1C during the two weeks study period. Data are represented as mean ± SEM, (n=5-8).
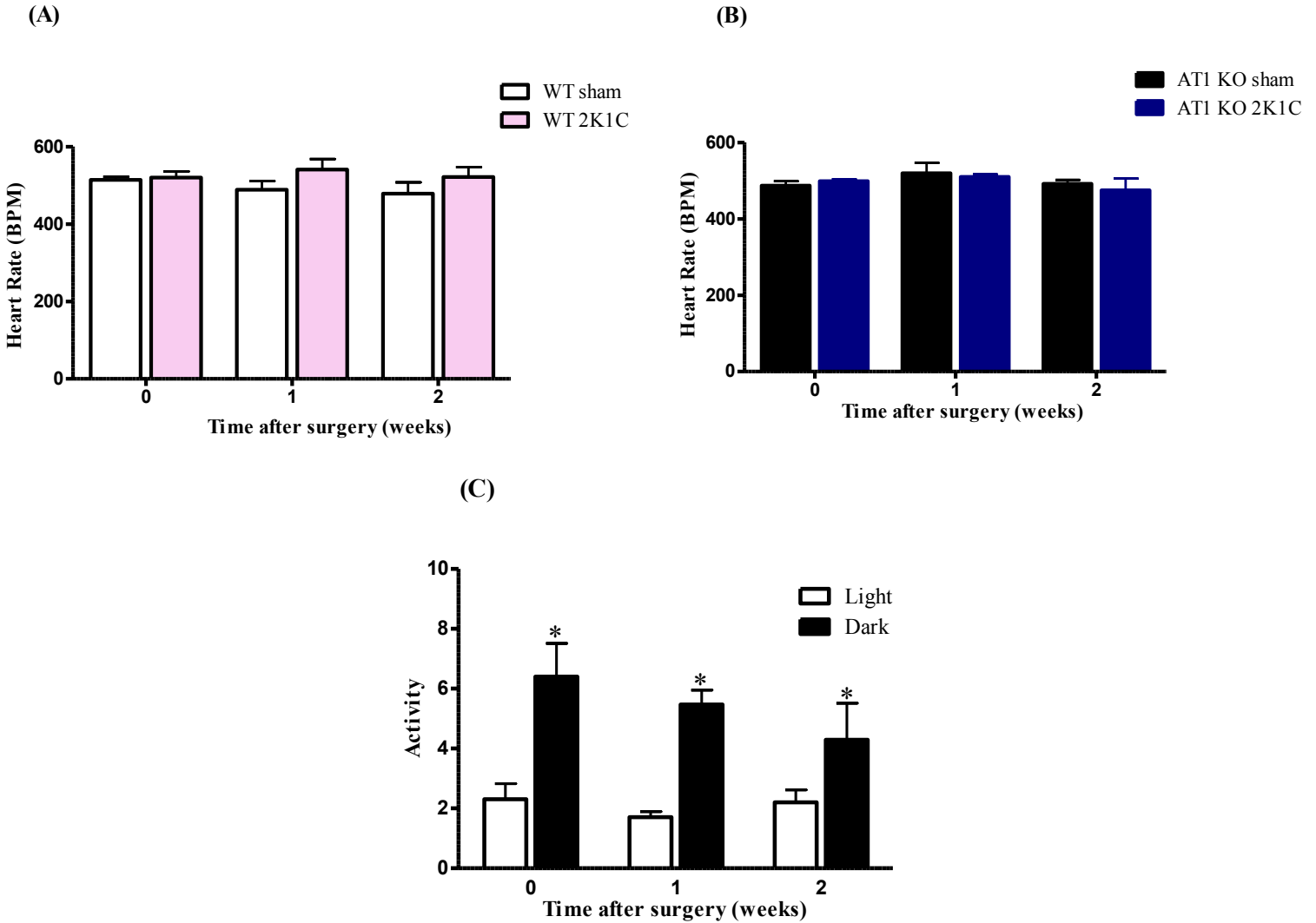
**Figure 7: Effect of 2K1C on heart rate and locomotor activity**

(A) Heart rate (HR) measurements in WT sham (white bars) and WT 2K1C (pink bars) (B) HR measurements in AT1 KO sham (black bars) and AT1 KO 2K1C (blue bars) (C) Locomotor activity in WT mice during light cycle (white bars) and dark cycle (black bars). Repeated measurements two-way ANOVA using a Bonferroni's posthoc test showed a significant increase in activity during the dark cycles compared to light cycles before (0) and after 2K1C. Data are represented as mean ± SEM, (n=5-8).

**(A)**



**(B)**
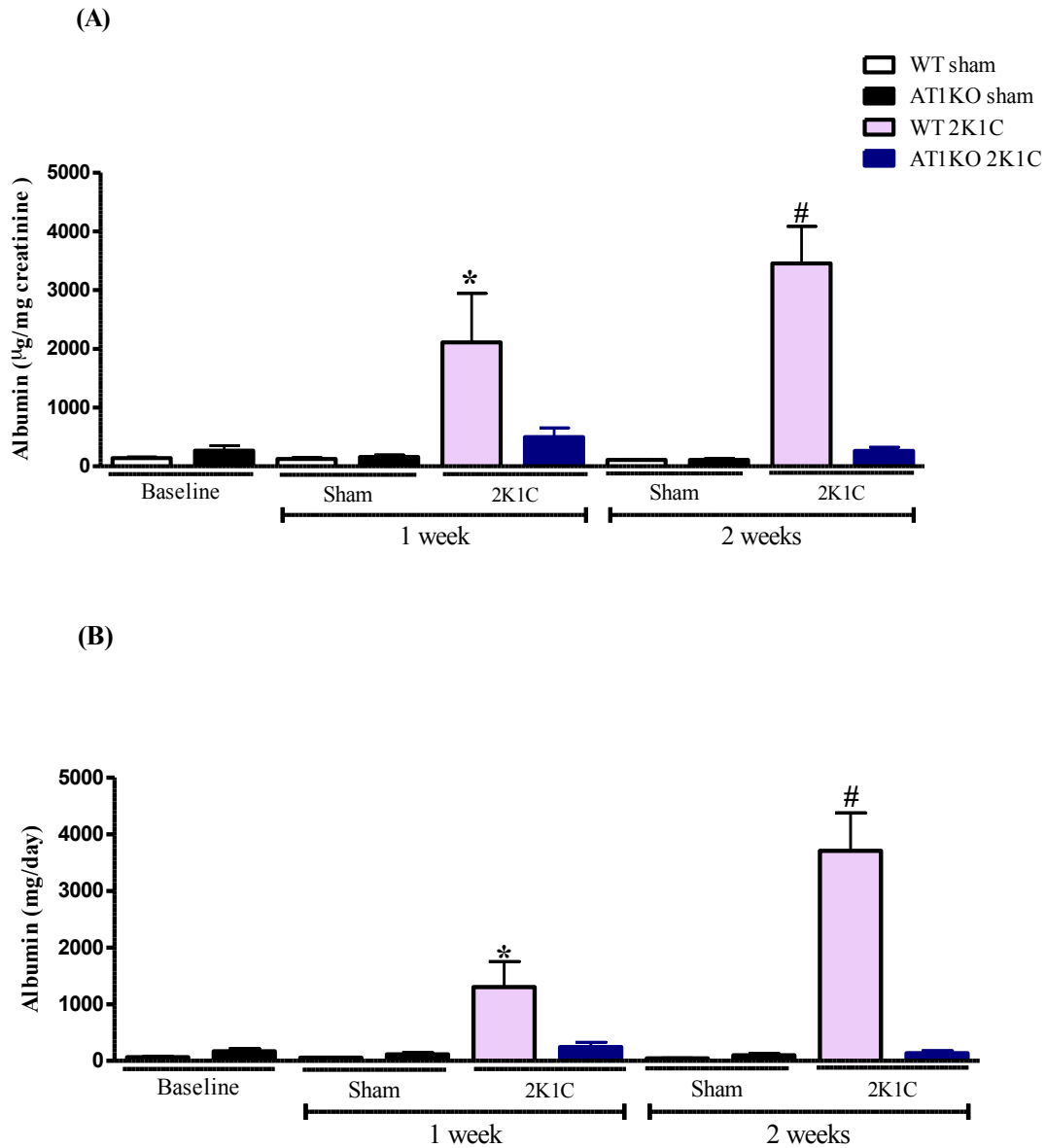


**Figure 8: Effect of 2K1C on urinary albumin excretion**

(A) Urinary Albumin excretion was expressed per creatinine in WT sham (white bar), AT1 KO sham (black bar), WT 2K1C (pink bar), and AT1 KO 2K1C (blue bar) (B) Urinary Albumin excretion per day. One-way ANOVA showed a significant increase of albumin excretion in WT 2K1C mice after one week ($^*p<0.001$) and two weeks by ($^\#p<0.0001$) compared to baseline, sham groups, and AT1 KO 2K1C mice. Data are represented as mean ± SEM, (n=5-10).

**Figure 9: Effect of 2K1C on renal ACE2 protein expression in WT mice**

Renal ACE2 protein expression in WT sham (white bar), unclipped kidney (pink bar), and clipped kidney (blue bar). Values were normalized by measurements of proportional intensity (NEP\β-actin) for each sample. One-way ANOVA showed a significant decreased in renal ACE2 expression in clipped kidney of 2K1C mice compared to sham and unclipped kidney of 2K1C mice ([**]$p<0.001$). Data are represented as mean ± SEM, (n=6).
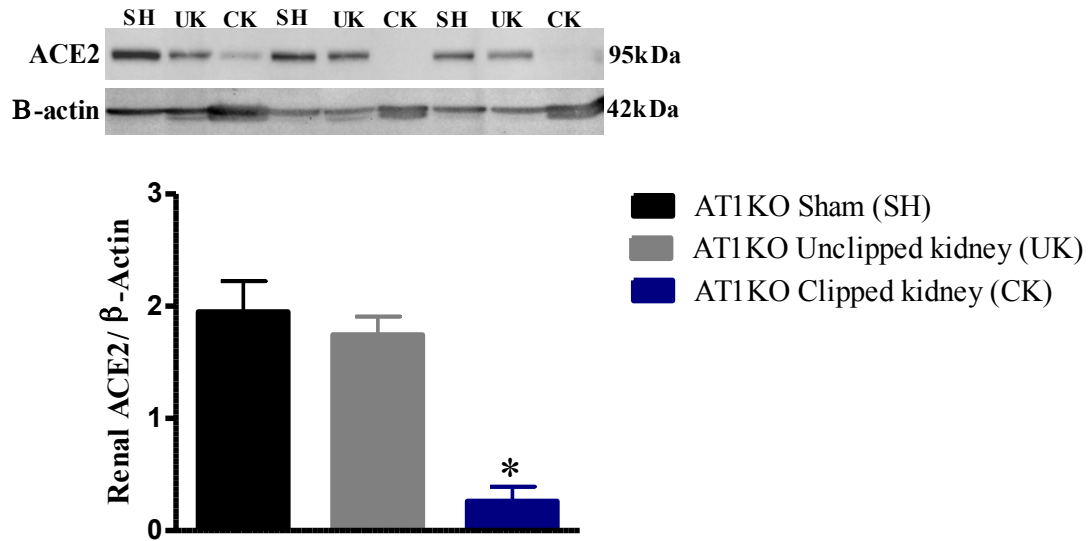
**Figure 10: Effect of 2K1C on renal ACE2 protein expression in AT1 KO mice**

Renal ACE2 protein expression in AT1 KO sham (black bar), unclipped kidney (gray bar), and clipped kidney (blue bar). Values were normalized by measurements of proportional intensity (NEP\β-actin) for each sample. One-way ANOVA showed a significant decreased in renal ACE2 expression in clipped kidney of 2K1C mice compared to sham and unclipped kidney of 2K1C mice ($^*p<0.0001$). Data are represented as mean ± SEM, (n=6).
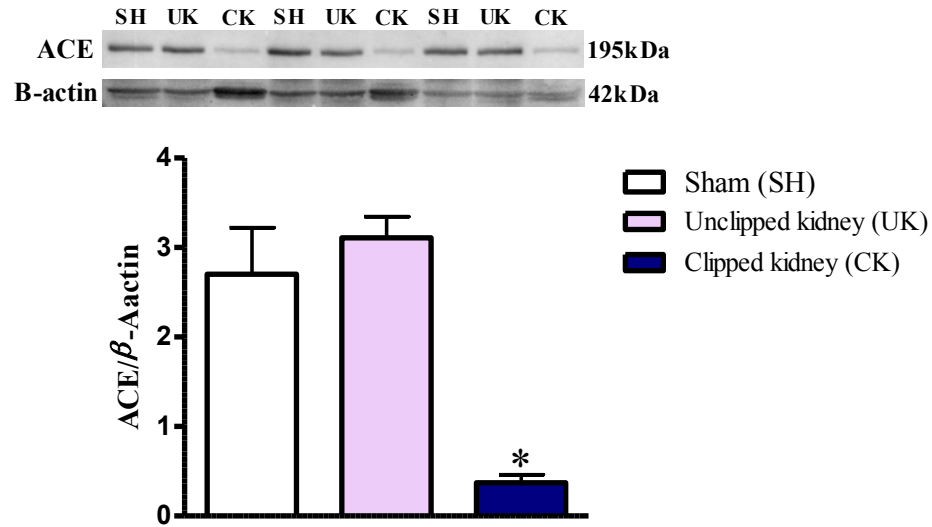
**Figure 11: Effect of 2K1C on renal ACE protein expression**

Renal ACE protein expression in WT sham (white bar), unclipped kidney (pink bar), and clipped kidney (blue bar). Values were normalized by measurements of proportional intensity (NEP\β-actin) for each sample. One-way ANOVA showed a significant decrease in renal ACE expression in clipped kidney of 2K1C mice compared to sham and unclipped kidneys of 2K1C mice ($^*p<0.0001$). Data are represented as mean ± SEM, (n=6).
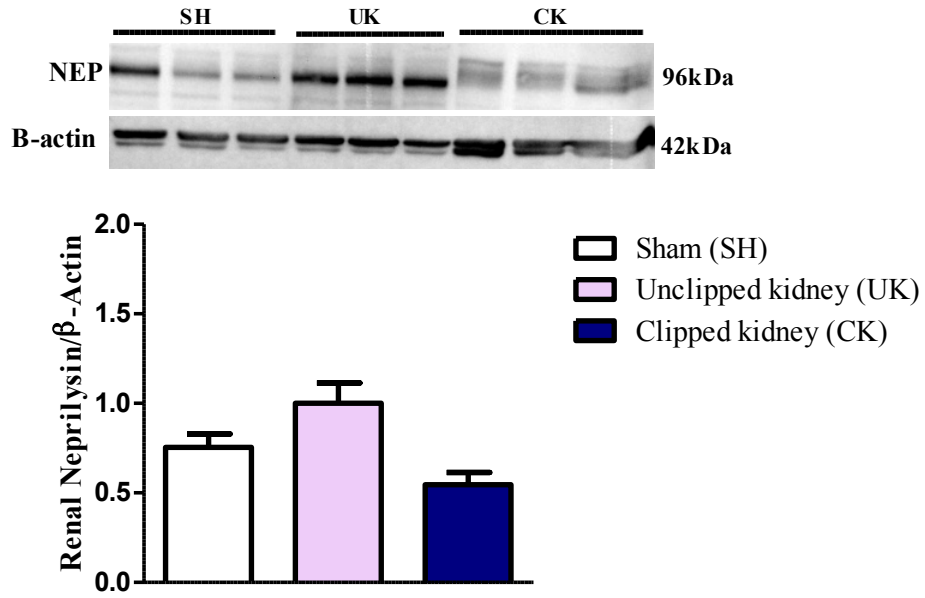
**Figure 12: Effect of 2K1C on renal NEP protein expression**

Renal NEP protein expression in WT sham (white bar), unclipped kidney (pink bar), and clipped kidney (blue bar). Values were normalized by measurements of proportional intensity (NEP\β-actin) for each sample. Renal NEP expression was decreased in clipped kidney of 2K1C mice compared to sham and unclipped kidney of 2K1C mice. Data are represented as mean ± SEM, (n=6).
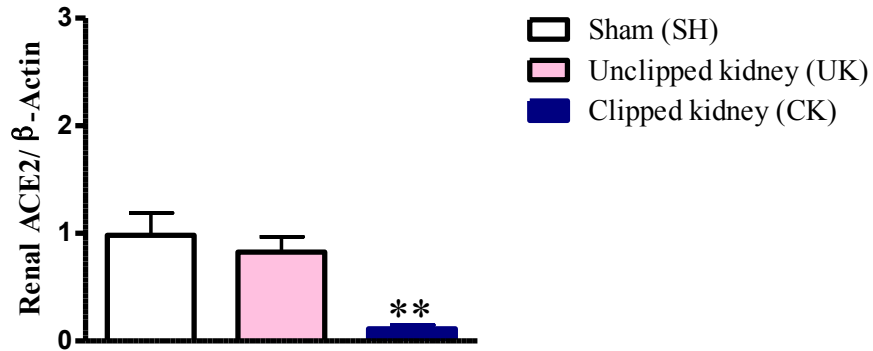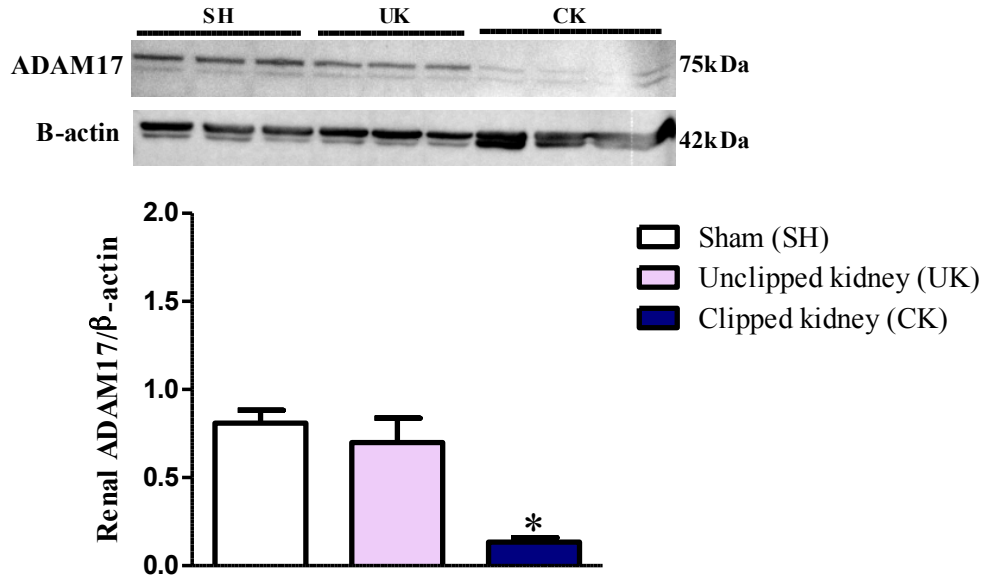
**Figure 13: Effect of 2K1C on renal ADAM17 protein expression**

Renal ADAM17 protein expression in WT sham (white bar), unclipped kidney (pink bar), and clipped kidney (blue bar). Values were normalized by measurements of proportional intensity (NEP\β-actin) for each sample. One-way ANOVA showed a significant decrease in renal ADAM17 expression in clipped kidney of 2K1C mice compared to sham ($^*p<0.0001$) and to unclipped kidney of 2K1C mice (by $^*p<0.001$). Data are represented as mean ± SEM, (n=6).
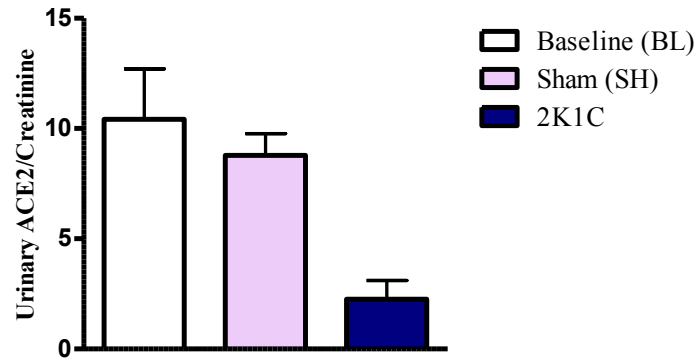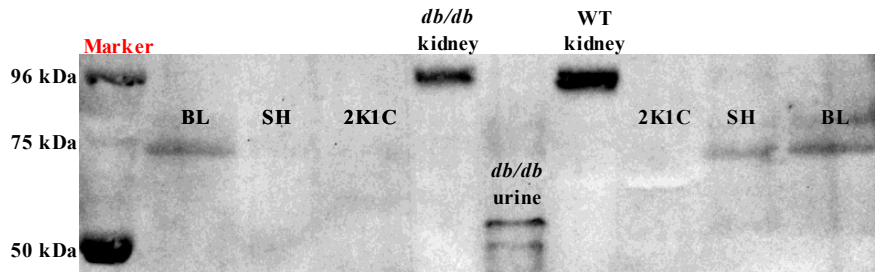
**Figure 14: Effect of 2K1C on urinary ACE2 excretion**

Urinary ACE2 excretion in WT baseline (white bar), sham (pink bar), and 2K1C (blue bar). Values were normalized by measurements of creatinine values for each sample. One-way ANOVA showed a decrease in urinary ACE2 excretion in 2K1C compared to baseline and sham groups. Data are represented as mean ± SEM, (n=5). Kidney lysate and urine samples from *db/db* diabetic mice and kidney lysate from WT mice were used as positive controls.
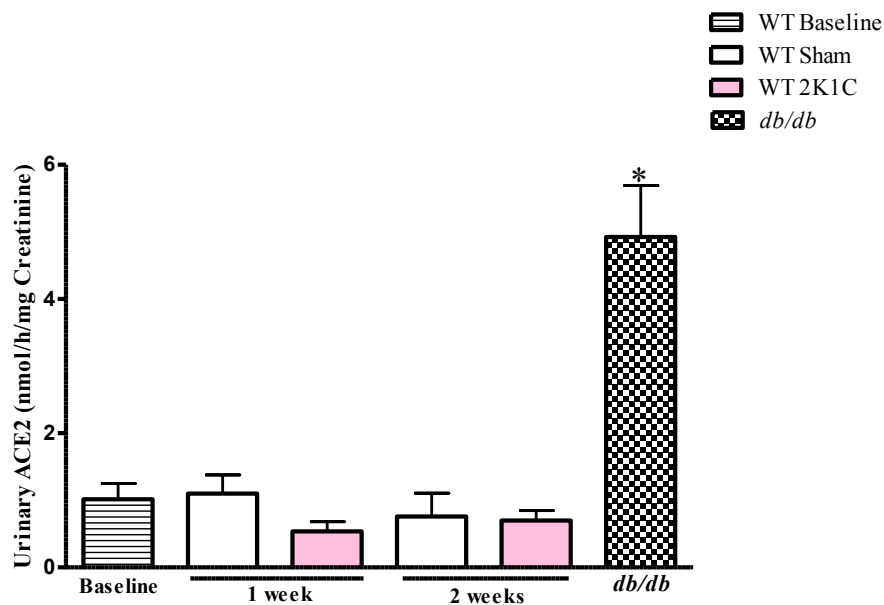
**Figure 15: Effect of 2K1C on urinary ACE2 activity in WT mice**

Urinary ACE2 activity in WT baseline (lined white bar), sham (white bar), and 2K1C (pink bar) 2K1C has no effect on the activity of urinary ACE2 enzyme in WT mice. Urine sample from *db/db* diabetic mice was used as positive control. One-way ANOVA showed a significant increase of urinary ACE2 activity in *db/db* compared to all groups at baseline and after 2K1C ([*]$p<0.0001$). Data are represented as mean ± SEM, (n=5-8).
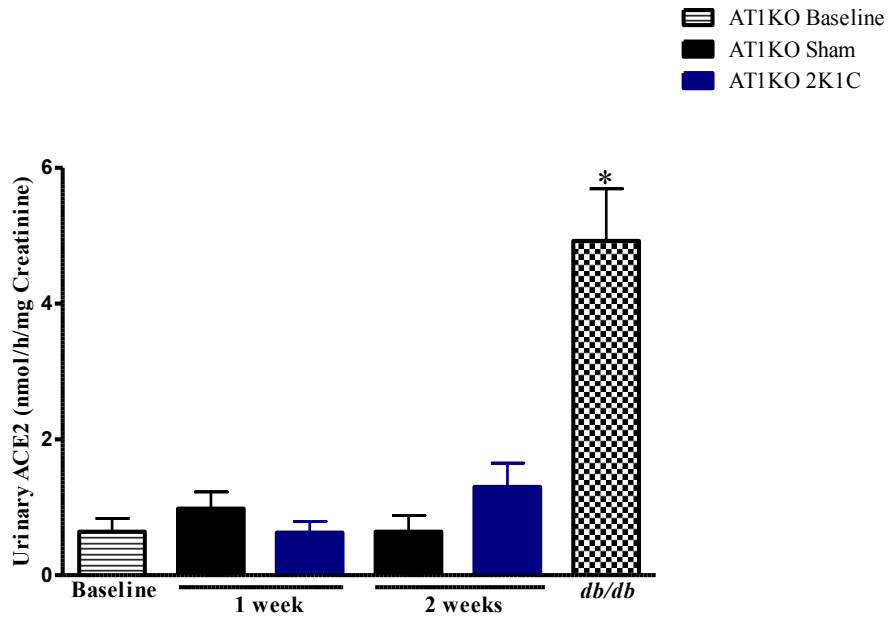
**Figure 16: Effect of 2K1C on urinary ACE2 activity in AT1 KO mice**

Urinary ACE2 activity in AT1 KO baseline (lined white bar), sham (black bar), and 2K1C mice (blue bar). 2K1C has no effect on the activity of urinary ACE2 enzyme in AT1 KO mice. Urine sample from *db/db* diabetic mice was used as positive control. One-way ANOVA showed a significant increase of urinary ACE2 activity in *db/db* compared to all groups at baseline and after 2K1C ($^*p<0.0001$). Data are represented as mean ± SEM, (n=5-8).
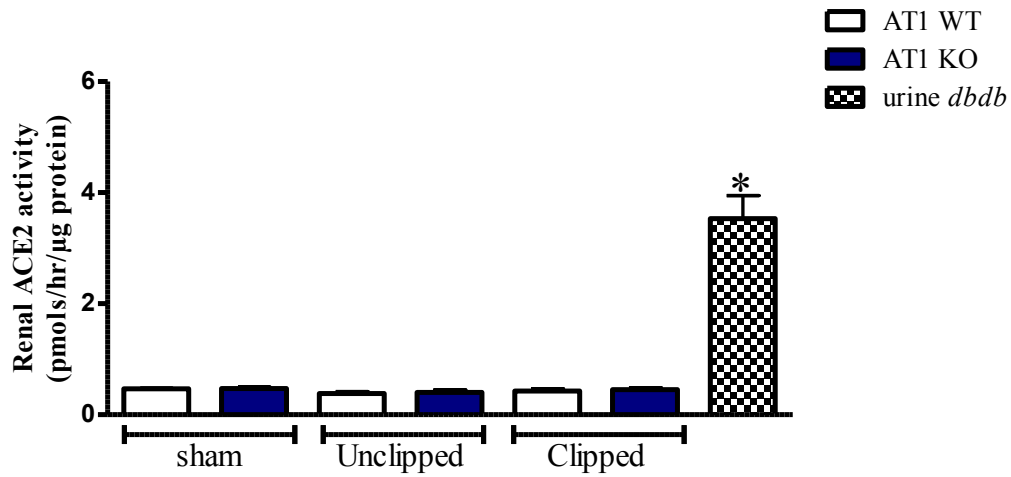
**Figure 17: Effect of 2K1C on renal ACE2 activity**

Renal ACE2 activity in WT (white bars) and AT1 KO (blue bars) for sham, unclipped, and clipped kidneys. 2K1C has no effect on renal ACE2 activity in WT and AT1 KO mice. Urine sample from *db/db* diabetic mice was used as positive control. One-way ANOVA showed a significant increase of ACE2 activity in diabetic *db/db* compared to all groups ($^*p<0.0001$). Data are represented as mean ± SEM, (n=6).
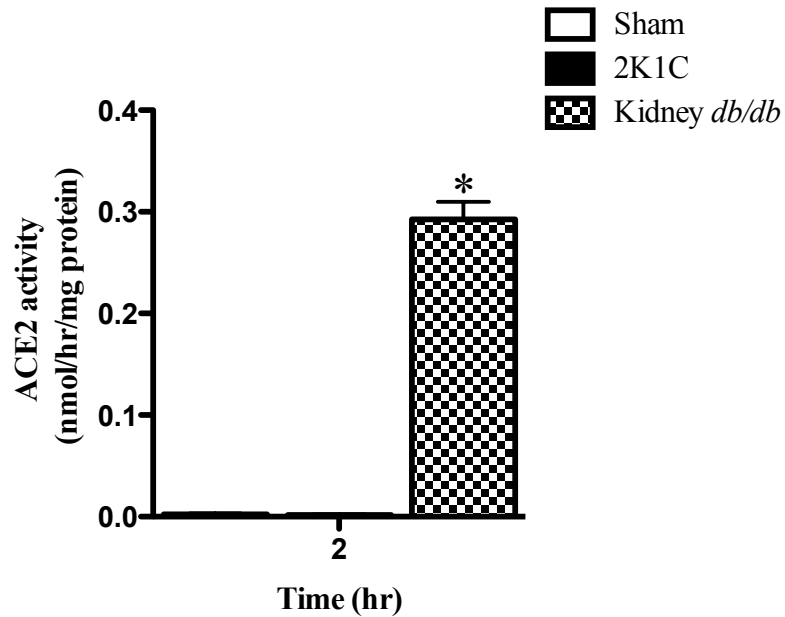
**Figure 18: Effect of 2K1C on plasma ACE2 activity**

Plasma ACE2 activity in WT sham (white bar) and WT 2K1C (black bar). Kidney lysate sample from *db/db* mice (dots white bar) was used as positive control. Plasma ACE2 activity in sham and 2K1C mice was not detectable. One-way ANOVA showed a significant increase of renal ACE2 in *db/db* compared to all groups ($^*p<0.0001$). Data are represented as mean ± SEM of group size (n=5-8).
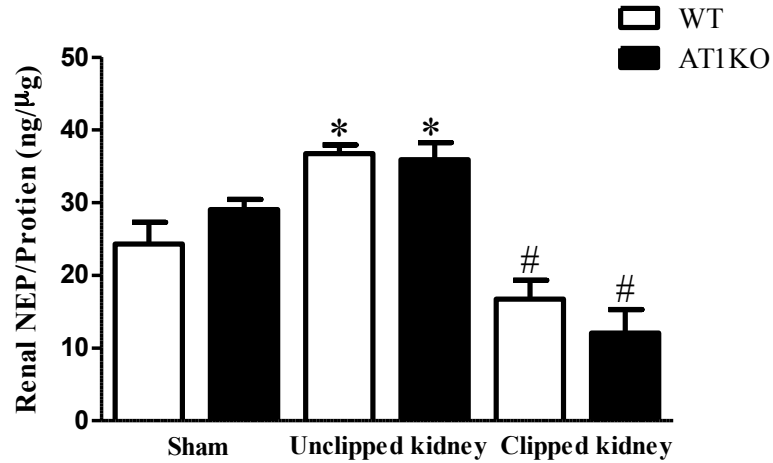
**Figure 19: Effect of 2K1C on renal NEP content**

Renal NEP content in WT (white bar) and AT1 KO (black bar). One-way ANOVA showed a significant decrease in renal NEP content in clipped kidney of 2K1C in WT and AT1 KO mice, compared to sham and unclipped kidney of 2K1C ($^{\#}p<0.0001$). The unclipped kidney had a significant increase of renal NEP content compared to sham kidneys ($^{*}p<0.05$). Data are represented as mean ± SEM of group size (n=5-7).

**(A)**



**(B)**



**(C)**
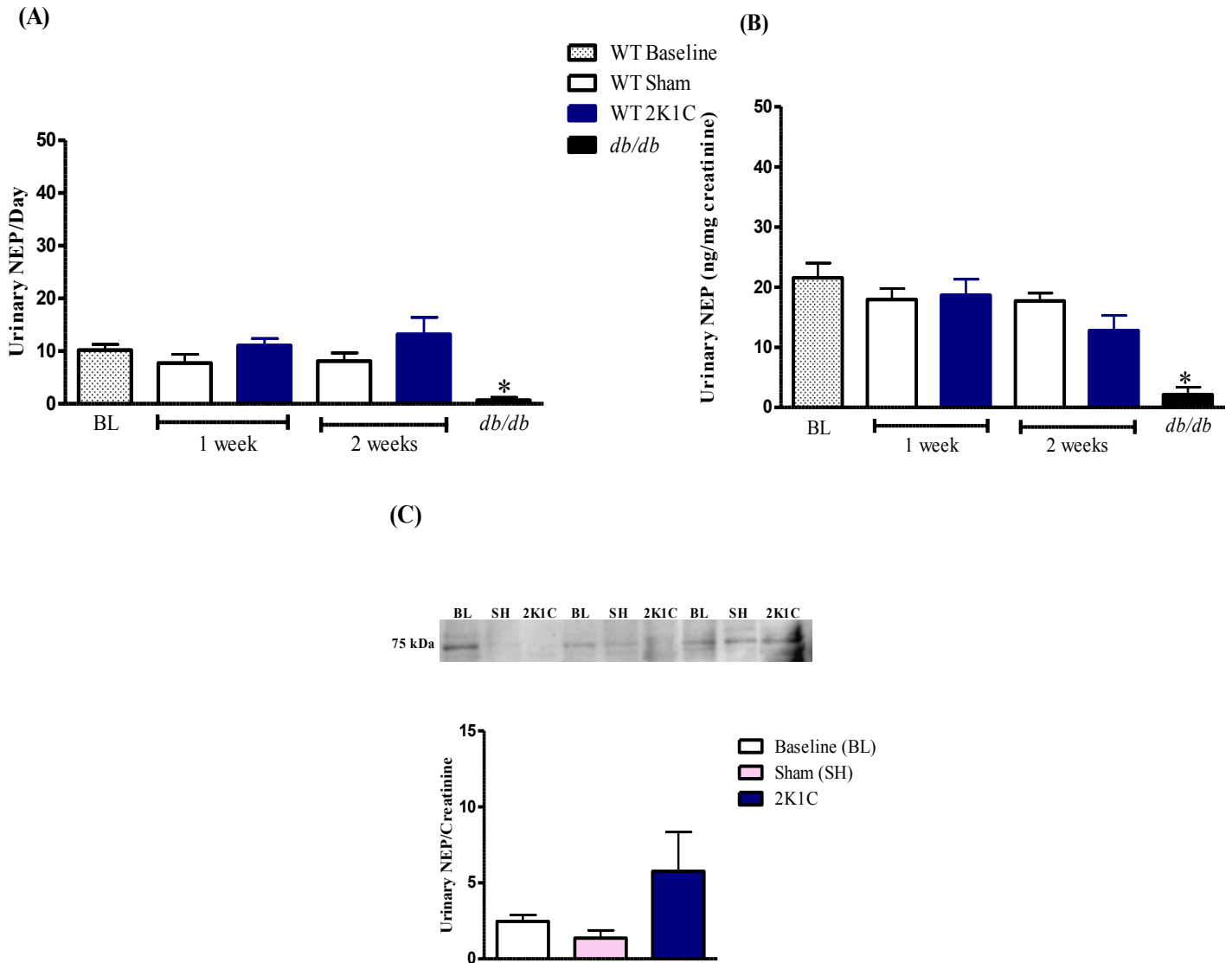


**Figure 20: Effect of 2K1C on urinary NEP content and expression**

Urinary NEP content was expressed per day (A) and per creatinine content (B) in WT sham (white bars), WT 2K1C (blue bars), and *db/db* diabetic mice (black bars). One-way ANOVA showed an increase in urinary NEP content in clipped kidney of 2K1C mice compared to baseline and sham groups, but was not significant. However, there was a significant decreased of urinary NEP in the diabetic mice compared to all groups ($^*p<0.0001$). (C) Urinary NEP expression in WT mice baseline (white bar), sham (pink bar), and 2K1C (blue bar). Values were normalized to creatinine values for each sample. One-way ANOVA showed an increase in urinary NEP expression but not significant in 2K1C compared to baseline and sham groups. Data are represented as mean ± SEM of group size (n=5-7).
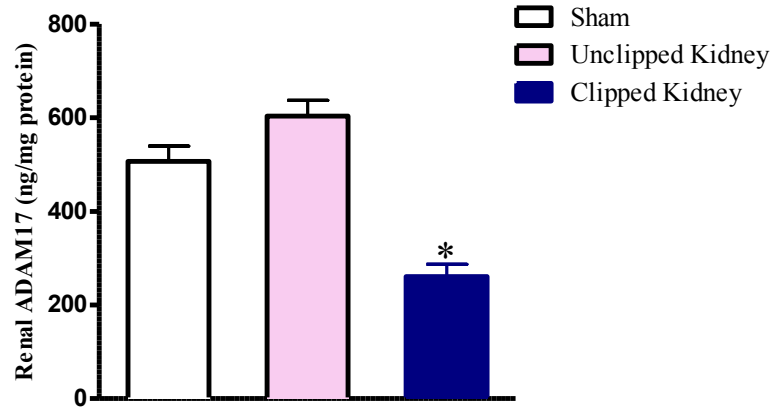
**Figure 21: Effect of 2K1C on renal ADAM17 content**

Renal ADAM17 content in WT sham (white bar), unclipped kidney (pink bar), and clipped kidney (blue bar). One-way ANOVA showed a significant decrease in renal ADAM17 content in clipped kidney of 2K1C compared to sham and unclipped kidney of 2K1C mice ($^*p<0.0001$). Data are represented as mean $\pm$ SEM of group size (n=6).
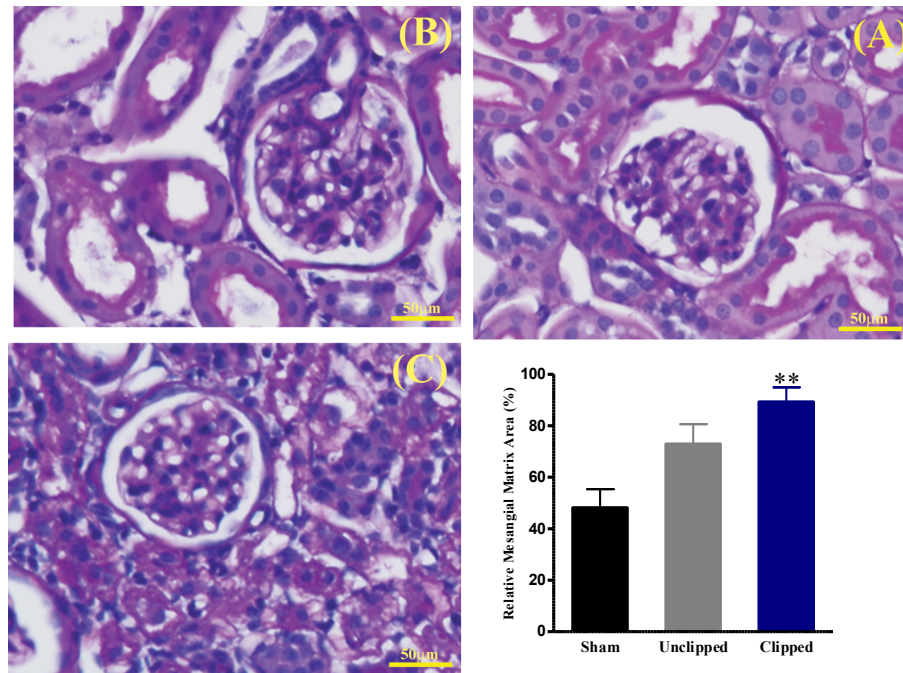
**Figure 22: PAS-stained kidney sections**

Representative images of PAS-stained kidney sections from (A) WT sham, (B) WT unclipped, and (C) WT clipped kidney of 2K1C mice at 40X magnification. One-way ANOVA showed a significant increase in the mesangial matrix expansion of clipped kidney of 2K1C (blue bar) compared to sham kidney (black bar) ($^{**}p<0.001$).
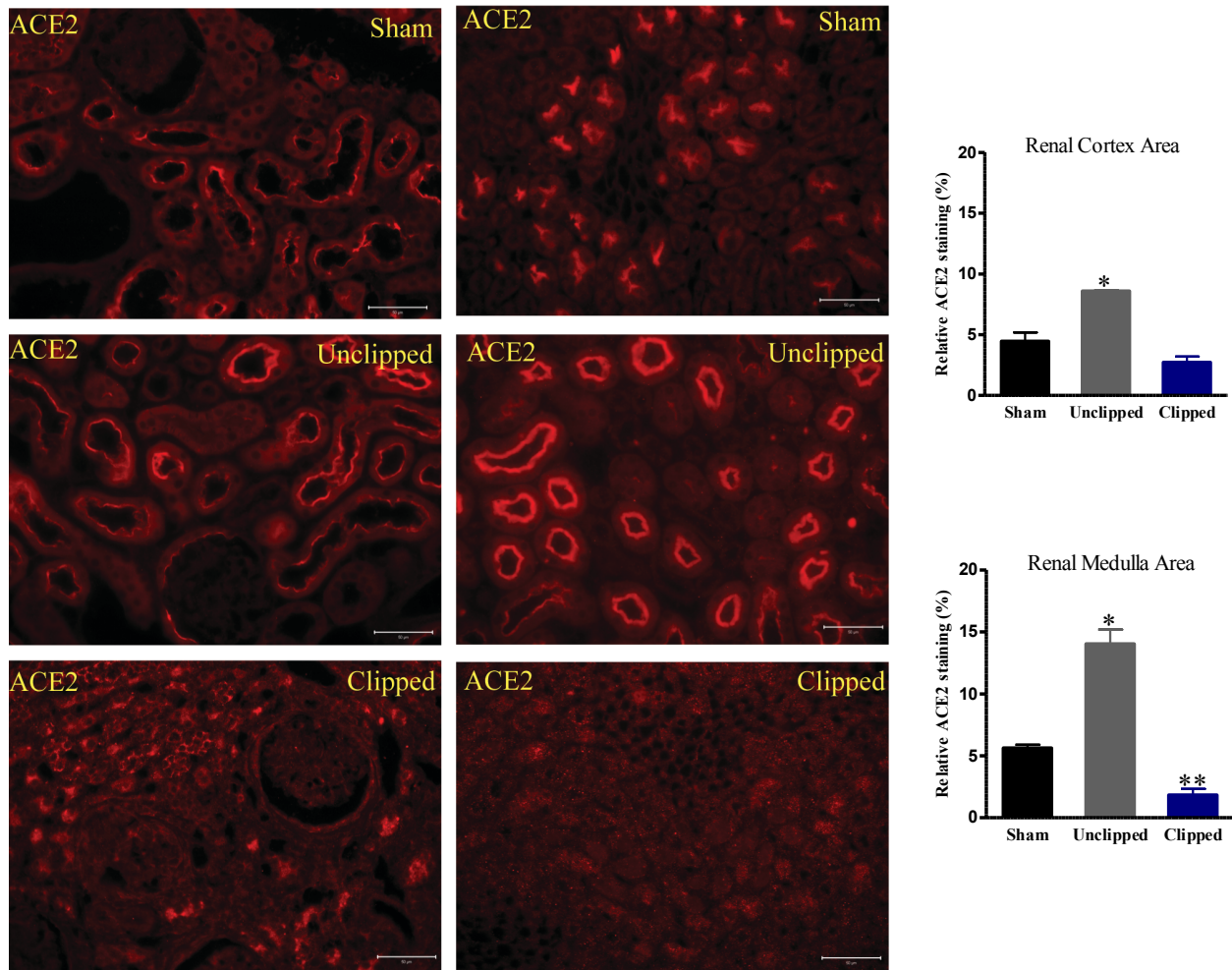
**Figure 23: Quantification of renal ACE2 immunostaining**

Representative images for ACE2 immunofluorescence staining taken from kidney sections of WT sham, unclipped, and clipped kidney of 2K1C mice at 40X magnification. The left panel shows the cortex area, while the right panel shows the medulla area. In the medulla, ACE2 shows a significant increase in the unclipped kidney (gray bar) compared to sham (black bar) ($^*p<0.05$) while the clipped kidney (blue bar) showed a significant decrease of ACE2 compared to unclipped kidney ($^{**}p<0.001$). In the cortex, ACE2 is highly stained in the distal and in the brush border of proximal tubules of sham and unclipped kidneys, and it is significantly increased in the unclipped compared to sham and clipped kidneys ($^*p<0.05$).
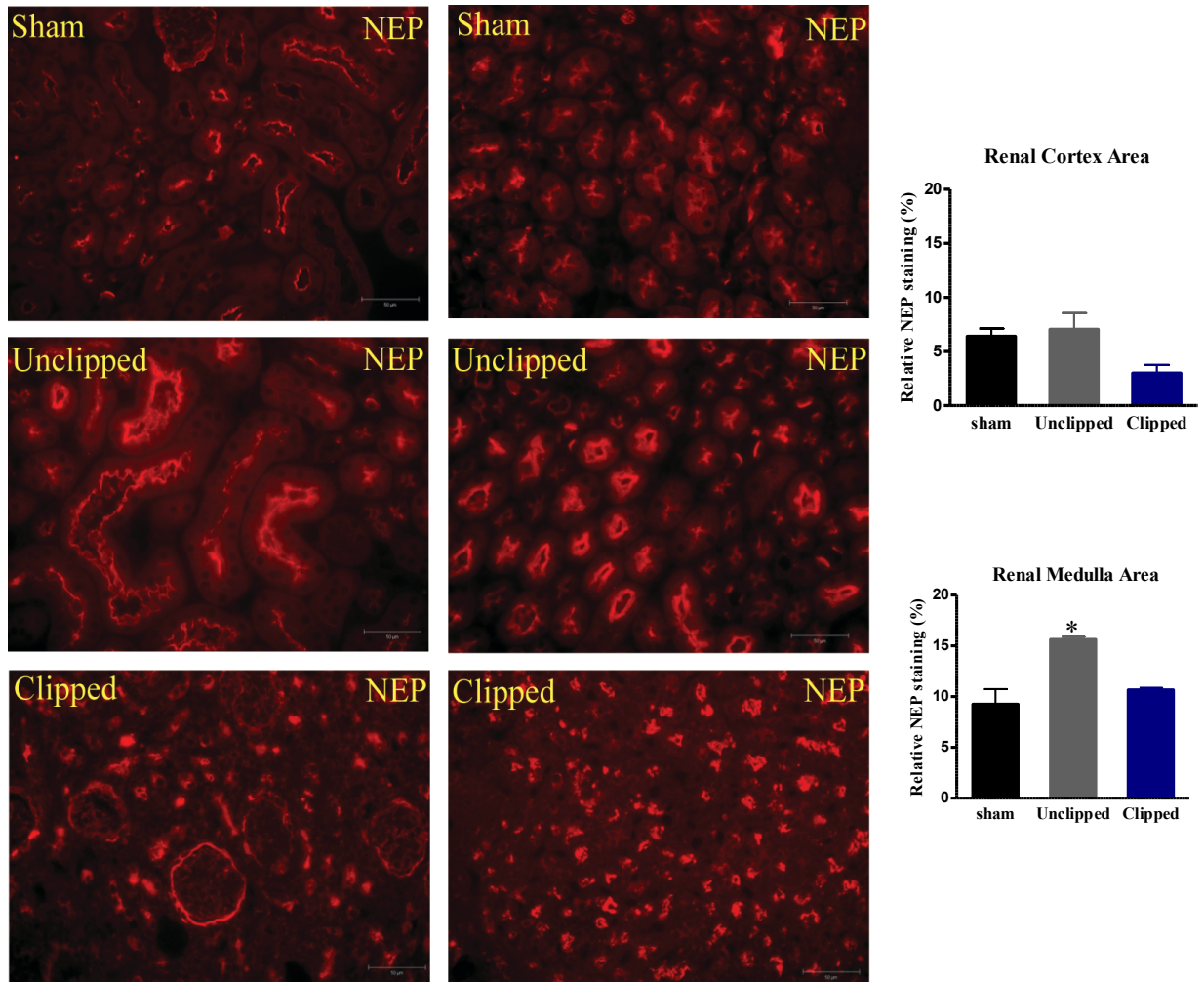
**Figure 24: Quantification of renal NEP immunostaining**

Representative images for NEP immunofluorescence staining taken from kidney sections of WT sham, unclipped, and clipped kidney of 2K1C mice at 40X magnification. The left panel shows the cortex area, while the right panel shows the medulla area. In the medulla, renal NEP is highly expressed in the unclipped kidney (gray bar) compared to sham one (black bar) ($^*p < 0.05$). It is extensively stained in Bowman's capsule of the glomeruli, and in the distal and the brush border of proximal tubules. In the cortex, renal NEP staining is decreased in clipped kidney compared to sham
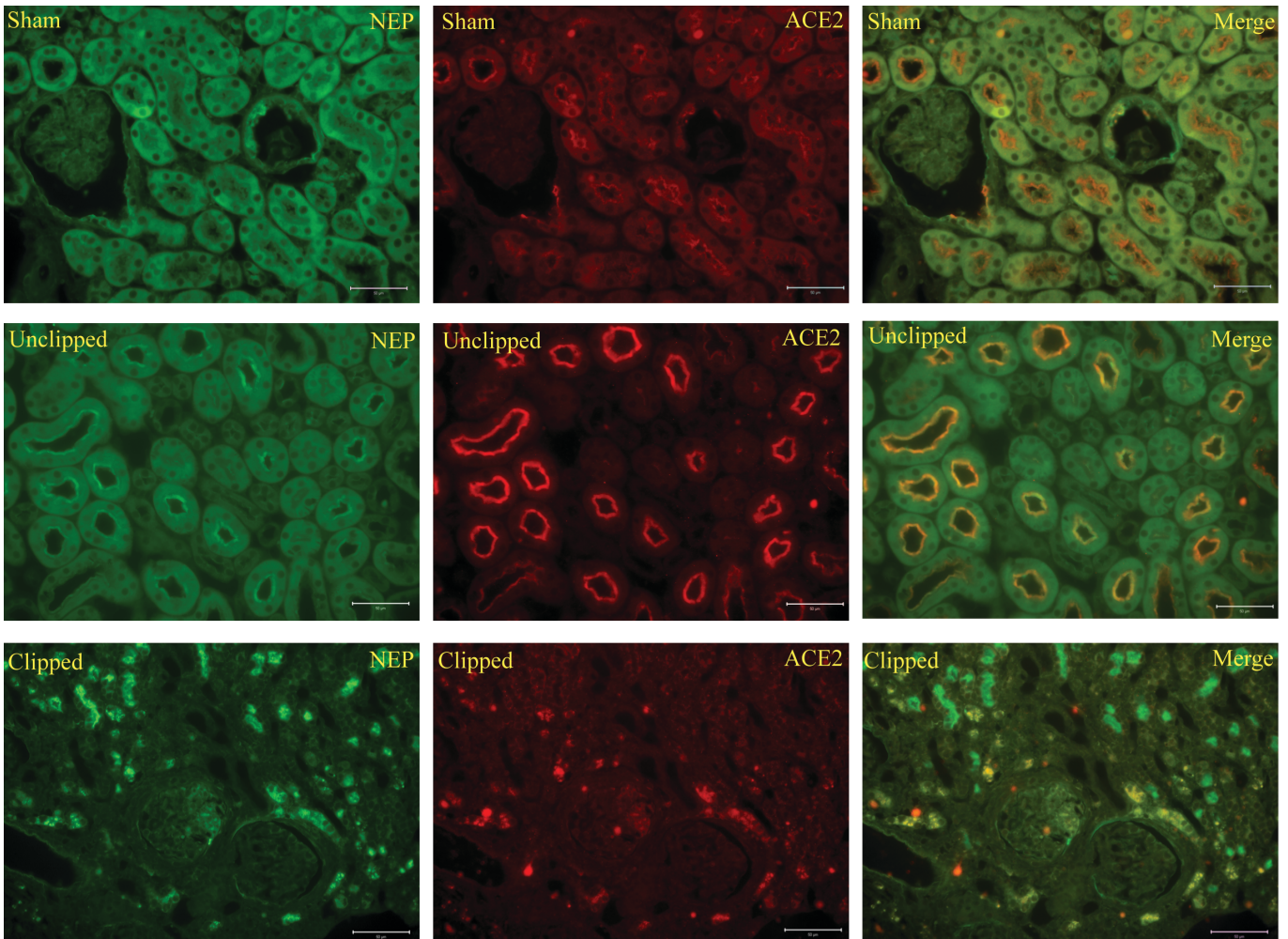
**Figure 25: Co-localization of renal NEP and ACE2 immunostaining**

Double immunofluorescence staining for NEP (green) and ACE2 (red) obtained from kidney sections of WT sham, unclipped, and clipped kidney of 2K1C mice at 40X magnification. Renal NEP and ACE2 were mainly co-localized in the distal and brush border of the proximal tubules. NEP signals were intensely stained in the renal capsule of the glomeruli.
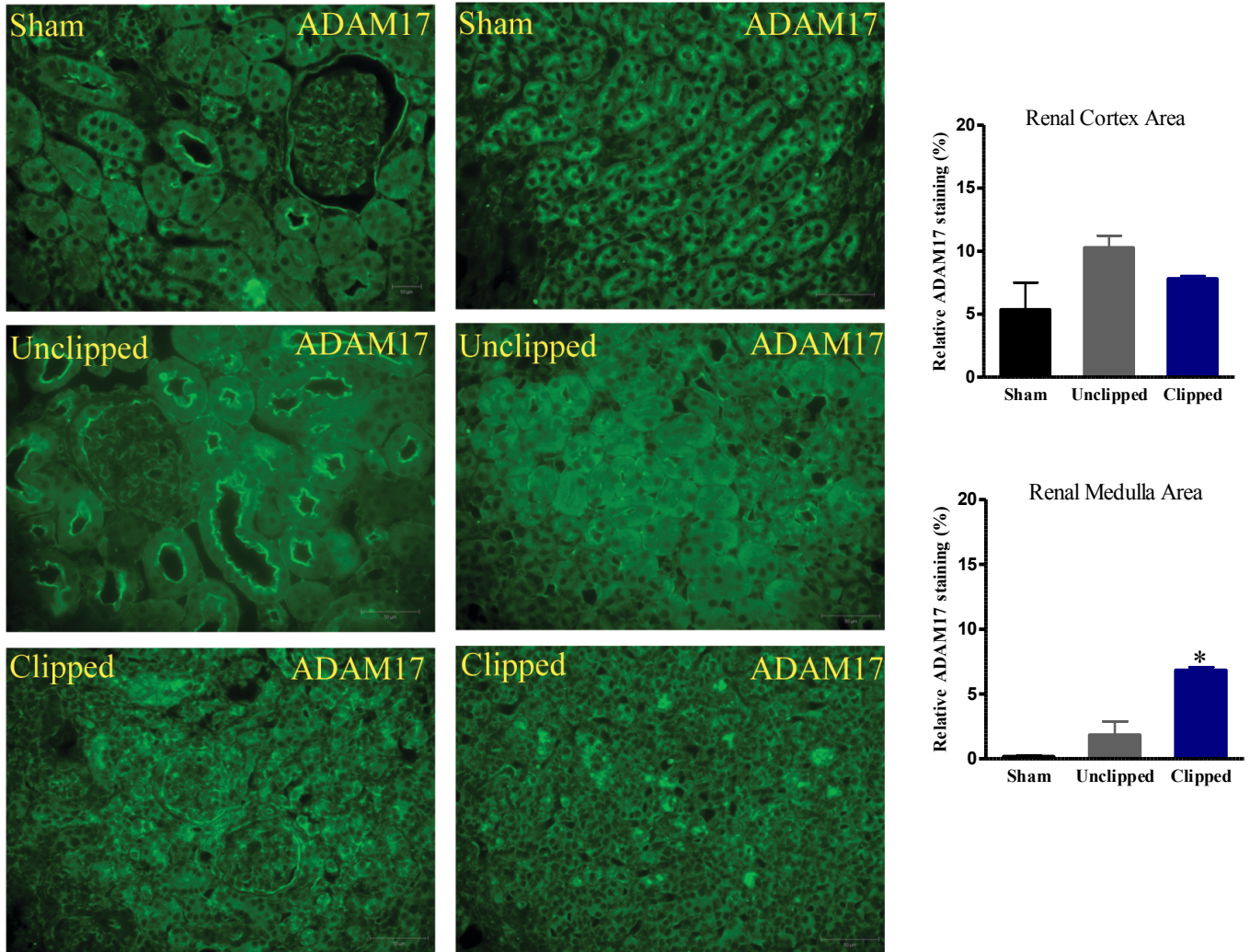
**Figure 26: Quantification of renal ADAM17 immunostaining**

Representative images for ADAM17 immunofluorescence staining taken from kidney sections of WT sham, unclipped, and clipped kidney of 2K1C mice at 40X magnification. The left panel shows the cortex area, while the right panel shows the medulla area. In the cortex, renal ADAM17 is highly expressed in the unclipped kidney compared to sham and clipped kidney, specifically in the glomerulus and the brush border of proximal tubules. However in the medulla, renal ADAM17 staining is significantly increased in clipped kidney compared to sham and unclipped ones ($^*p<0.05$).
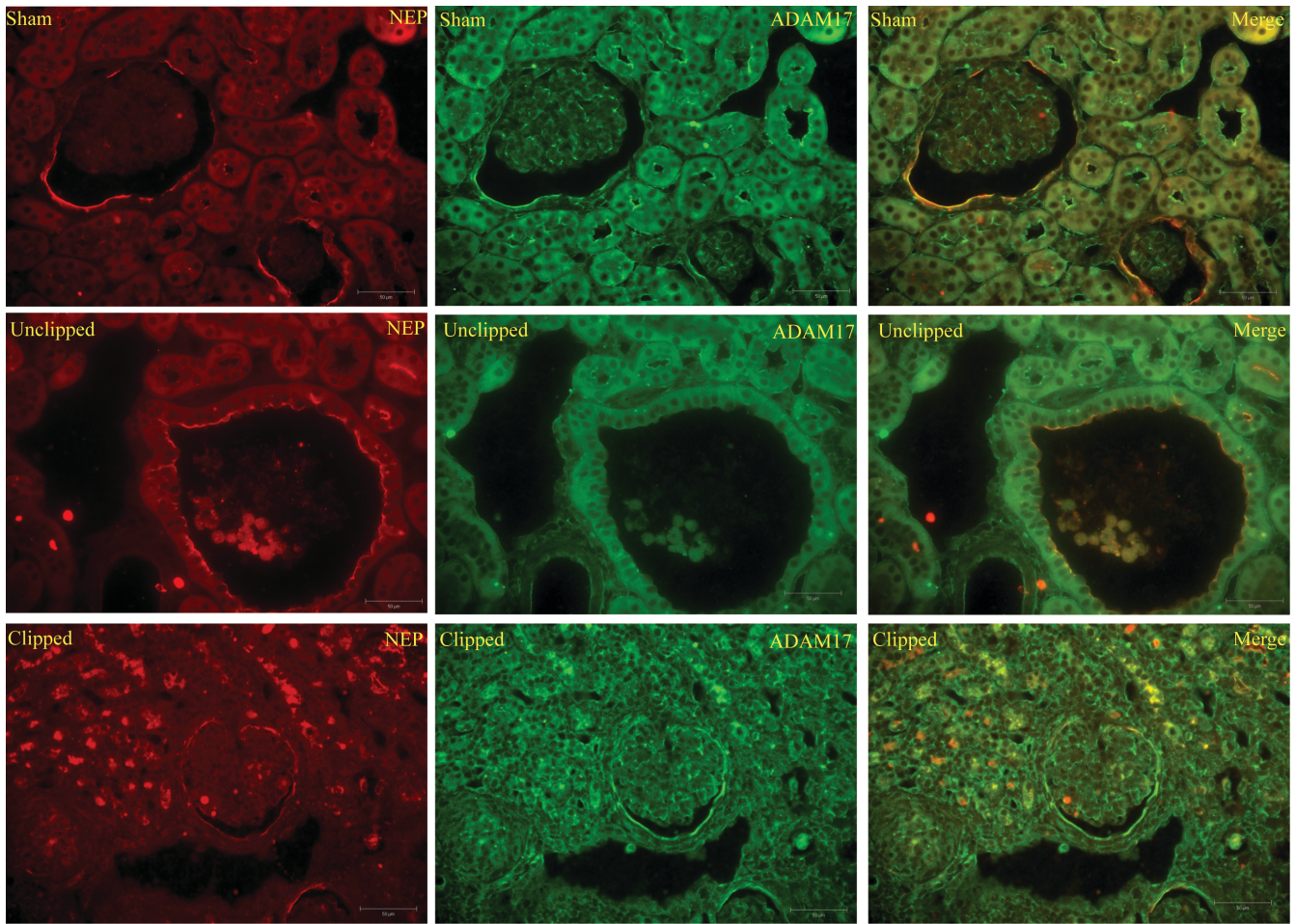
**Figure 27: Co-localization of renal NEP and ADAM17 immunostaining**

Double immunofluorescence staining for NEP (red) and ADAM17 (green) obtained from kidney sections of WT sham, unclipped, and clipped kidney of 2K1C mice at 40X magnification. Renal NEP and ADAM17 were mainly co-localized in the distal and brush border of the proximal tubules. NEP signals were intensely stained in the renal capsule of the glomeruli, while ADAM17 signals were stained in the glomerulus area.

# 4. RESULTS

## 4.1. Effect of renal artery clipping (2K1C) on general physiological parameters

Male Wild Type (WT) and AT1$_A$R Knockout (AT1KO) mice were implanted with radio-telemetry and after one week of recovery, renovascular hypertension was induced in AT1KO and WT mice by placing renal clips in the left renal artery. After one week recovery, general physiological parameters were measured for 2 weeks.

Table (2) shows the general physiological parameters, which were measured on alternating days for all groups of mice.

A) Food intake and body weight: 2K1C had no effect on total body weight (Figure 1) or food intake (Figure 2) in neither the WT nor the AT1KO mice during the study time period.

B) Water intake: In WT mice, 2K1C significantly increased water intake compared to age-matched WT sham-operated mice (Figure 3A and table 2). However, in AT1KO, there was no difference in water intake between AT1KO 2K1C and AT1KO sham-operated mice (Figure 3B). At the baseline, when no surgical procedures were applied, we observed a significant increase in water intake of AT1KO mice compared to WT mice ($^{**}p<0.0001$, Figure 3C), while after 2K1C, water intake was decreased in AT1KO mice compared to WT but was not statistically significant (Figure 3D). In addition, water intake of sham-operated groups showed no difference compared to the baseline groups.

C) Urine output: Figure 4, panel (A) represents the daily urine output of WT mice. It shows that urine output significantly increased one week after induction of 2K1C ($^{#}p<0.0001$, table 2) and persisted during the second week of 2K1C ($^{*}p<0.05$, table 2) compared to baseline and sham-operated groups. However, in AT1KO mice,

2K1C increased urine output, but was not statistically significant (Figure 4B). The daily urine volumes were significantly higher in AT1KO mice compared with age-matched WT mice (Figure 4C, **$p<0.001$).

D) Cardiovascular parameters and locomotor activity: Radio-telemetry was used to measure 24h (mean arterial blood pressure, heart rate, locomotor activity) every other day during the two weeks of the study. We observed a significant increase in MAP after the unilateral renal artery (clipping) during the two weeks in WT 2K1C compared to age-matched WT sham-operated mice (Figure 5, *$p<0.05$). As expected, MAP was significantly decreased in AT1KO mice compared with WT mice before (0) and even after induction of 2K1C renovascular hypertension (Figure 6A, *$p<0.05$). Interestingly, in AT1KO mice, no change in MAP was observed after 2K1C compared with controls (AT1KO sham-operated) (Figure 6B). In addition, there was no statistical difference in MAP between the light and dark cycles in the 2K1C mice. In Figure 7, heart rate measurements showed no statistically difference between sham and 2K1C of WT (Figure 7A) and AT1KO (Figure 7B) during the two weeks of the study period. However, the locomotor activity in WT was significantly increased during the dark cycle when compared to the light one (figure 7C) at the baseline (0) and after the weeks of the 2K1C.

## 4.2. Evaluation of renal function in 2K1C mice

The 24-hour urine samples were collected to determine an estimate of the renal function, albumin excretion, creatinine excretion, and total protein.

A) Urinary Albumin excretion: Figure 8A shows the urinary albumin excretion, corrected per unit of creatinine, increased from the first day of the unilateral renal

artery clipping (2K1C) throughout the study period. After the first week, the urinary albumin excretion level was significantly increased in 2K1C mice ($^*p<0.001$), and was increased even more at the end of the study period in the second week ($^\#p<0.0001$) when compared with the baseline and sham-operated animals. Panel B in the figure shows the albumin excretion rate expressed per day. The results are consistent with findings obtained using albumin creatinine ratio. As expected, AT1KO mice showed no difference in albumin excretion after 2K1C was applied during the whole study period. Also, there was no statistical difference in urinary albumin excretion in sham-operated mice for both WT and AT1KO mice.

B) Urinary creatinine excretion: WT 2K1C mice excreted significantly less creatinine when compared with age-matched WT sham-operated mice (table 2, $^*p<0.0001$), while in AT1KO mice there was no significant difference between sham and 2K1C (table 2).

C) Urinary protein excretion: In agreement with albuminuria results, total urinary protein excretion per day in WT mice was significantly higher in 2K1C mice compared with their baseline (10.3±2.4 mg/day vs. 6.9±0.6 mg/day) and sham-operated mice (10.3±2.4 mg/day vs. 5.2±1.9 mg/day).

### 4.3. Determination of renal proteins expression in sham and 2K1C mice

Renal ACE2, NEP, and ADAM 17 protein expressions were quantified by Western blot analysis in kidney lysate samples.

A) Renal ACE2 protein expression: A single band for ACE2 was detected at molecular weight ~95 kDa. This result is consistent with the full length of ACE2 reported by others (Chodavarapu et al., 2013; Somineni et al., 2014). In WT mice,

renal ACE2 expression was significantly decreased in the clipped kidneys compared to their unclipped and sham kidneys (Figure 9, $^{**}p<0.001$). In AT1KO mice, renal ACE2 was also significantly reduced in the clipped kidneys compared to the unclipped and sham kidneys (Figure 10, $^{*}p<0.0001$).

B) Renal ACE protein expression: In the kidney, a single band for ACE was seen at molecular weight ~195 kDa. In agreement with previous studies (Navar et al., 1998; Navar et al., 1995), renal ACE expression was significantly decreased in the clipped kidney compared to unclipped or sham kidneys (Figure 11, $^{*}p<0.0001$).

C) Renal NEP protein expression: In the kidney, a single band for NEP was detected at a molecular weight ~96 kDa in clipped, unclipped, and sham-kidneys (Figure 12). There was a decrease in renal NEP protein expression in clipped kidneys compared to sham or unclipped kidneys, however, ANOVA didn't show statistical significance. (Figure 12).

D) Renal ADAM17 protein expression: In the kidney, a band for ADAM17 was detected at a molecular weight ~75 kDa. The clipped kidney of 2K1C showed a significant decrease of renal ADAM17 expression compared to unclipped and sham kidneys (Figure 13, $^{*}p<0.001$).

## 4.4. Determination of urinary protein expression in sham and 2K1C mice

In this project, we propose to use urinary ACE2 and NEP as index of intrarenal status. To investigate the effect of 2KIC on shedding of renal RAS into urine, Western blot analysis was used to determine the urinary protein expression of ACE, ACE2 and NEP in 24-hour urine samples collected after 2 weeks of induction of 2K1C renovascular hypertension and sham operation.

A) Urinary ACE2 protein expression: Immunoreactive band for urinary ACE2 expression was seen as a smaller molecular weight ~75 kDa band compared to the full length immunoreactive band seen in kidney (Figure 14). Urinary ACE2 was decreased in 2K1C mice compared to sham and baseline (Figure 14).

B) Urinary NEP protein expression: Urinary NEP expression was detected at approximately ~75 kDa, which is smaller in size compare to renal NEP band. Urinary NEP protein was increased in 2K1C mice compared to baseline and sham-operated ones, but it was not statistically significant (Figure 20C).

## 4.5. Effect of 2K1C on ACE2 activity

To investigate the effect of 2K1C on renal and urinary ACE2 shedding, urinary ACE2 activity was measure in urine and kidney lysate. ACE2 activity was determined using the fluorogenic substrate Mca-APK (Dnp) in 2-10 μl urine samples (2-5 μg creatinine) in the presence of the ACE inhibitor, lisinopril.

A) Urinary ACE2 activity: 2K1C had no effect on urinary ACE2 activity in WT mice (Figure 15) or AT1KO mice (Figure 16) during the two weeks of the study. Urine samples from *db/db* type 2 diabetic mice were used as positive controls, and as expected showed a significant increase in urinary ACE2 activity compared to all groups ($^*p<0.0001$).

B) Renal ACE2 activity: Thirty-thirty five micrograms (30-35 μg) from kidney lysate were added in the presence of the ACE inhibitor, lisinopril. 2K1C had no effect on the activity of renal ACE2 in WT and AT1KO, during the two weeks of the study. Urine samples from *db/db* diabetic mice were used as a positive control group, and it showed a significant increase of ACE2 activity compared to all groups (Figure 17, $^*p<0.0001$).

C) Plasma ACE2 activity: To investigate the impact of 2K1C on circulating ACE2, plasma ACE2 activity was measured in plasma samples (1-0 µl). Plasma ACE2 activity was not detected in WT and AT1KO regardless of procedures as shown in Figure 18. Kidney lysates from *db/db* diabetic mice were used as a control, and it showed a significant increase of renal ACE2 activity compared to all groups (figure 18, $^*p<0.0001$).

## 4.6. Quantification of renal and urinary proteins content in sham and 2K1C mice

A) NEP content: ELISA was used to quantify renal and urinary NEP. The ELISA data demonstrate significantly low levels of renal NEP content in the clipped kidney of 2K1C group in both WT and AT1KO mice compared to the unclipped and sham kidneys (Figure 19, $^\#p<0.0001$). Moreover, there was a significant increase in renal NEP in the WT and AT1KO unclipped kidneys when compared with WT sham kidneys (Figure 19, $^*p<0.05$).

B) However, during the two weeks of the study period, urinary NEP protein excretion rate (expressed/day) was increased in WT 2K1C mice compared to baseline and sham groups, but it was not statically significant (Figure 20A). Urinary NEP protein content showed no significant difference between sham and 2K1C mice when normalized to creatinine (Figure 20B). Urine samples from *db/db* diabetic mice were used as a control, showing that urinary NEP content was significantly decreased in the diabetic mice compared to baseline, sham-operated, and 2K1C (Figure 20, $^*p<0.0001$).

C) ADAM17 content: Renal ADAM17 contents were measured by ELISA. In WT, renal ADAM17 content was significantly decreased in 2K1C clipped kidney compared with 2K1C unclipped and sham kidneys (figure 21, $^*p<0.0001$).

## 4.7. Histopathology of sham and 2K1C kidneys

### PAS staining

To evaluate the renal pathology of clipped, unclipped, and sham (control) kidneys, PAS staining was performed. A significant mesangial expansion was observed in kidneys of 2K1C, in the clipped and the unclipped one, compared to the sham kidney (Figure 22).

### Immunohistochemistry for renal NEP and ADAM17 expression

Immunofluorescence staining was used to determine the localization of ACE2 protein expression in kidney sections from sham, unclipped 2K1C, and clipped 2K1C. As shown in figure 23, renal ACE2 is located in the brush border of the proximal tubules in the sham and unclipped kidneys. Renal ACE2 is significantly decreased in the clipped kidney in both the cortex and medulla when compared to the sham and unclipped one. In the medulla of the unclipped kidney, renal ACE2 was significantly increased compared to the same region of sham or clipped kidneys.

Renal NEP immunofluorescence staining was observed in all regions of sham, unclipped, and clipped kidneys. NEP was mainly expressed in the Bowman's capsule of the glomeruli and in distal and the brush border of the proximal convoluted tubules. NEP was highly expressed in sham and unclipped kidneys compared to the clipped one (Figure 24, left panel). NEP was also highly expressed in the medulla area in the unclipped kidney compared to the sham and clipped one (Figure 24, right panel, $^{*}p<0.05$).

Figure 26 represents renal ADAM17 immunofluorescence staining, and shows that the unclipped kidney has higher staining in comparison to the sham kidney. In the medulla

region, ADAM17 staining was observed in the clipped kidney, while no detection of ADAM17 staining was found in the sham and unclipped kidney sections (Figure 26, right panel).

Figure 25 represents the double immunofluorescence staining for NEP (green) and ACE2 (red) proteins in kidney sections of sham, unclipped, and clipped kidneys, in order to determine the co-localization of these proteins. NEP and ACE2 were predominantly co-localized in the distal and the brush border of proximal tubules, in both kidneys (clipped and unclipped) of 2K1C. NEP was found also in the Bowman's capsule of the glomeruli. Moreover, ADAM17 (green) and NEP (red) proteins were mainly co-localized to the distal and the brush border of the proximal tubules in the sham kidney as well as in the unclipped one (Figure 27). Furthermore, strong staining was found for NEP in the renal capsule of the glomeruli, while ADAM17 staining was observed in the glomerulus area (Figure 27).

## 5. DISCUSSION

In summary, we demonstrate here that 2K1C-induced hypertension is associated with decreased renal ACE2 and NEP. Deleting $AT1_AR$ in mice is associated with decreased MAP and attenuation of microalbuminuria in 2K1C model of renovascular hypertension, suggesting a critical role for $AT1_AR$ in hypertension and renal physiology. The functional significance of arterial and renovascular hypertension on renal and urinary ACE2 has not yet been fully elucidated. However, in this study, deleting $AT1_AR$ didn't protect against the decreased renal ACE2 protein expression and activity in the clipped kidney.

According to the Kidney International Supplements, diabetes mellitus and hypertension remain the two main causes of CKD, and eventually lead to ESRD (Kidney International Supplements, 2013). Previous study stated that the shedding of renal ACE2 in the urine in type 2 diabetic *db\db* mouse model could act as a potential early biomarker for diabetic nephropathy (Chodavarapu et al., 2013). In addition, Salem et al. conducted a similar study, using the diabetic Akita mouse model, where they suggested that increased urinary ACE2 might be associated with increase renal ACE2 shedding through renal ADAM17 activation (Salem et al., 2014). However, studies on the activation of intrarenal RAS components in hypertensive conditions such as the 2K1C, a model of Ang II-dependent hypertension (Navar & Harrison-Bernard, 2000), and their involvement in renoprotection are limited.

In the current study, we tested the hypotheses that in the 2K1C renovascular hypertension mice model, there is an increase of renal ACE2 shedding into the urine, which may contribute to the progression of CKD. In the 2K1C model, the reduction in the renal perfusion pressure caused by unilateral renal artery stenosis led to an increase of renin

synthesis, which in turn increased the circulation of the vasoconstrictor Ang II (Kobori et al., 2007). The G protein-coupled receptor AT1R is the predominant receptor responsible for the mediation of most of the Ang II biological functions (Oliverio & Coffman, 2000). The action of Ang II is antagonized via the formation of the vasodilator Ang (1-7), which is mainly produced by ACE2 or NEP (Rice et al., 2004). Binding of Ang (1-7) with either AT2R (Santos et al., 2003) or mas receptor (Batlle et al., 2012) could lead to a decline of proliferation, fibrosis, hypertrophy, and the vasoconstriction effect (Bian et al., 2013). Treatment of the hypertensive transgenic Ren2 rats with AT1R blocker (ARB) increases renal ACE2 and NEP expression, suggesting a contribution of renal RAS components to renoprotective effects (Whaley-Connell et al., 2006). Regulation of blood pressure and cardiovascular function via ACE2 has been reported recently in the protection from neurogenic hypertension, and has been proposed as a new target in the treatment of hypertension and cardiovascular disease (Feng et al., 2010). A recent study demonstrated the protective role of Ang (1-7) in the attenuation of systemic hypertension and renal injury in diabetic mice model, as well as normalizing renal ACE2 and mas receptor expression (Shi et al., 2014). Due to the physiological antagonistic effects of mas and AT1R, researchers have been focusing on the important balance of these receptors as a therapeutic target in vascular diseases (Kostenis et al., 2005).

Our first aim was to investigate the role of $AT1_A R$ in blood pressure mediation in the 2K1C and its impact on renal and urinary ACE2 and NEP protein. Several studies have focused on the most active peptide of the intrarenal RAS, Ang II, due to its critical role in hypertension and renal injury (Belova, 2000). The elevation of blood pressure is mainly mediated by the vasoconstrictor Ang II when it binds to its receptor, AT1R (de et al.,

2000). In our study, we found a significant decrease in blood pressure in $AT1_A$ KO mice at baseline, which is in agreement with previous studies (Gurley et al., 2011; Cervenka et al., 2002; Grobe et al., 2015b; Oliverio et al., 2000). As expected, WT mice showed a robust increase in MAP after placement of the unilateral renal clip when compared to sham-operated mice throughout the two weeks of the study. In contrast, we observed no change in MAP of $AT1_A$KO mice after 2K1C during day and night. The MAP remained significantly low in $AT1_A$KO after 2K1C. These data provide strong evidence in support of the notion that the AT1R plays a role in the pathogenesis of renovascular hypertension, which corresponds with a study that reported the predominate role of $AT1_A$R in the 2K1C model (Cervenka et al., 2008). To further clarify the role of RAS in 2K1C, Salguero eat al. demonstrated that renovascular hypertension in WT mice was significantly reduced by treatment with ACE inhibitor, and ARB (Salguero et al., 2008) In addition, Crowley and coworkers stated in one study that AT1R has a direct effect on Ang II resulting in high blood pressure and that inhibition of renal AT1R would be more effective in the protection from cardiac hypertrophy compared with inhibition of the AT1R in the heart (Crowley et al., 2006).

Our second finding of this study showed that renal ACE2 protein expression was significantly decreased in the clipped kidney of 2K1C in both WT and $AT1_A$R KO mice, compared with the unclipped and sham-operated kidneys (Figure 9&10). In contrast, there was a significant increase of renal ACE2 staining in the unclipped kidney compared to the sham and clipped ones. This discrepancy between the expression of renal ACE2 using western blot and the immunostaining of kidney sections could be due to the difference of antibodies used, in which a goat anti-ACE2 from R&D was used for Western analysis,

while a rabbit anti-ACE2 from Sigma was used for the immunofluorescence data. However, immunostaining for ACE2 in the medulla demonstrated a significant decrease in the clipped kidney in WT mice, which agrees with the western blot analysis. Using *db/db* mice, we demonstrated a significant increase in urinary ACE2 activity (Figure 15,16 &17), which agrees with previous studies that diabetic nephropathy was associated with an increase in urinary ACE2 (Chodavarapu et al., 2013; Somineni et al., 2014). One of our original hypotheses that, renovascular hypertension will also increase the shedding of ACE2 and contribute to the onset of albuminuria. However, there was no alteration of renal and urinary ACE2 activity in 2K1C –induced renovascular hypertension.

In mouse model of diabetes nephropathy, there was evidence of increased renal ACE2 and increased shedding of ACE2 into urine (Batlle et al., 2012). However, data demonstrated protective role of $AT1_AR$ on albuminuria and against 2K1C –induced hypertension. These effects are independent of ACE2, since we found that $AT1_AR$ has no effect on the expression of either renal or urinary ACE2 protein when compared with WT mice. Similarly, in $AT1_AKO$ mice, 2K1C has no effect on renal and urinary ACE2 activity.

One of our previous reports stated that plasma ACE2 activity was undetectable in *db/db* diabetic mice (Chodavarapu et al., 2013); similarly, we found plasma ACE2 undetectable in 2K1C and sham-operated mice. Thus, the down-regulation of urinary ACE2 protein excretion that we observed in 2K1C mice may reflect the renal ACE2 status. These findings of decreased renal and urinary ACE2 contradict both our hypothesis and previous findings in diabetic animals, but it is in agreement with a study on subtotal nephrectomized (STNx) rats (an acute kidney injury model), stating that renal ACE2 activity is reduced in acute kidney injury, which contributes to the development of CKD

(Velkoska et al., 2010). We could conclude from these results that presence of high levels of urinary ACE2 in diabetic models and low levels in 2K1C and STNx models may indicate the specificity of ACE2 as a biomarker for the prognosis of renal injury in diabetes.

ADAM17 is a member of a disintegrin and metalloprotease family (White, 2003) and it is involved in the ectodomain shedding of several transmembrane proteins (Black et al., 1997). The ectodomain shedding of ACE2 has been shown in various cell lines such as human proximal 323 tubular HK-2 cells (Salem et al., 2014), human hepatoma cell lines Huh1 and Huh7 (Ford et al., 2013; Salem et al., 2014), and mouse proximal tubular primary cells (Xiao et al., 2014). One of our recent studies using COS7 cell line demonstrated the crucial role of ADAM17 in the ectodomain shedding of renal ACE2 (Grobe et al., 2015a). In addition, renal ADAM17 was highly expressed in Akita and *db/db* diabetic mice models, and mediated the increase shedding of renal ACE2 (Salem et al., 2014; Somineni et al., 2014). Corresponding to the low levels of protein expression of ACE2 in 2K1C, we also found a significant decrease of renal ADAM17 content and protein expression in the clipped kidney of 2K1C mice.

NEP has been reported in several studies to be involved in the degradation of various active peptides such as Ang I, bradykinin, natriuretic peptides (Judge et al., 2014), and beta amyloid (Park et al., 2013). Previous studies showed a decrease in renal and urinary NEP in the *db/db* diabetic mice, which was normalized by treatment with rosiglitazone (Chodavarapu et al., 2012; Chodavarapu et al., 2011). In this study, we also investigate whether NEP is modulated by 2K1C. The data demonstrated a significant decrease of renal NEP protein expression in the clipped kidney of 2K1C compared to the unclipped

and sham-operated kidneys. Grobe et al. showed downregulation of renal protease prolyl carboxypeptidase (PRCP) expression and activity, another enzyme that forms Ang (1-7) from Ang II, in the clipped kidney of 2K1C compared to the unclipped and control kidneys (Grobe et al., 2015b). Since 2K1C is a high renin model, therefore we anticipated less production of Ang 1-7 via NEP and this could lead to kidney injury. However, the unclipped kidney of 2K1C showed a significant increase of renal NEP content in both mice WT and $AT1_A$ KO compared to the sham, and this could be a feedback mechanism from high level of Ang II.

Our data showed a decrease in urinary NEP in *db/db* mice, which agrees with previous study. However, 2K1C did not decrease urinary NEP shedding compared to their sham controls (Figure 20). Again, this highlights the difference in kidney injury between diabetes and 2K1C-induced hypertension. Since in the cancer field, researchers have proposed NEP, also known as CD10, as a distinguished biomarker for various malignant tumors such as prostate cancer (Ho et al., 2013), renal neoplasm (Avery et al., 2000), and malignant melanoma (Bilalovic et al., 2004). Therefore, in this study we could use NEP as a biomarker for kidney injury in hypertensive conditions.

Until now, urinary albumin excretion and estimated glomerular filtration rate (eGFR) were the gold standard indicators used diagnostically for the assessment of CKD (Currie et al., 2014). However, there are some cases presented with renal dysfunction but without microalbuminuria, indicating the insensitivity of these biomarkers, especially in the early stages of renal disease (Lee, 2014). Therefore, there is a need for an early sensitive biomarker in the detection of CKD.

Our third aim of the present study was to evaluate the urinary albumin excretion in this model as an indicator for kidney dysfunction, as well as to determine if $AT1_AR$ has a role in the development of CKD in hypertensive subjects. We found that urinary albumin excretion per day in WT mice is increased after the unilateral renal clip was placed from the first day of the 2K1C surgery. This increase in urinary albumin excretion was significant after one week (Figure 8, *$p$<0.001) when compared with the baseline and sham-operated groups. At the end of the study (after two weeks), the 2K1C mice excreted more urinary albumin than the first week of the surgery, which indicates albuminuria is increased with the progression of age (Figure 8, #$p$<0.0001). However, 2K1C did not change the urinary albumin excretion in the $AT1_AKO$ mice during the whole study period. This data determines the pivotal role of activation AT1R in the development of albuminuria in the renovascular hypertension model.

## 6. CONCLUSION

Our results support the notion that $AT1_AR$ plays a crucial role in the 2K1C model of renovascular hypertension, and deleting of this receptor attenuates albuminuria in this model. Moreover, our findings suggest a possible link between ADAM17 and ACE2 shedding in 2K1C mice. Since previous studies demonstrated the significance shedding of renal ACE2 into the urine of diabetic mice models, while no effect was observed in renal ACE2 shedding in renovascular hypertensive model, which suggests the specificity of ACE2 as a biomarker for diabetic nephropathy. Downregulation of renal ACE2 and NEP in the clipped kidney of 2K1C may deteriorate kidney injury and renoprotective effects via impaired formation of Ang (1-7) from Ang II and Ang I respectively.

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