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Original Article

Dysregulation of kidney proteases in the pathogenesis of hypertension following unilateral nephrectomy in juvenile mice

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Abstract: Background: Unliteral nephrectomy (UNX) results in the reduction of kidney mass. The remaining kidney undergoes compensatory renal growth via hypertrophy of the glomeruli and renal tubules to maintain a normal glomerular filtration rate (GFR). These compensatory mechanisms result in increased capillary pressure and glomerular hyperfiltration to increase single nephron GFR. Over time, hyperfiltration may lead to kidney scarring and the development of hypertension. Objectives: The first objective of this study was to test the hypothesis that a 50% reduction in functioning nephrons in juvenile mice leads to increased blood pressure over a 24-hour phase. The second objective was to test the hypothesis that UNX leads to changes in the expression and activity of kidney proteases in juvenile mice. Methods: Eight male C57B6 juvenile wild-type mice were subject to UNX and an equal number of mice were subject to sham (SH) surgery. Metabolic cage studies were performed for 5 weeks to collect urine produced during the inactive and active phases. Blood pressure was measured using the tail cuff method twice weekly and tail blood was collected on different days during the inactive or active phase of each animal. The mice were euthanized at the age of 9 weeks. Western blotting and immunohistochemistry were performed to investigate changes in renal protein expression of various cathepsins and renal kallikrein 1 (KLK1) between the two groups. Protease activity assays were performed using kidney lysates and urine samples from each group. Results: Compared to the SH group, UNX mice showed a persistent increase in blood pressure at week 3 which progressed toward the end of the study at week 5 of age. Cathepsin B, D, and S expression and activity were up-regulated in kidney cortex lysates from UNX mice compared to the SH control group. KLK1 protein expression was down-regulated and urinary nitric oxide excretion was decreased in UNX mice compared to the SH control group. Conclusion: UNX results in the development of persistent and progressive hypertension. Down-regulation of KLK1 and up-regulation of various cathepsins may contribute to the development of hypertension via multiple mechanisms including a decrease in nitric oxide (NO) production.

Keywords: Unilateral nephrectomy, hypertension, kidney proteases, cathepsins, renal kallikrein

Introduction

Unilateral nephrectomy (UNX) is a surgical procedure that involves the removal of one kidney, resulting in a reduction in kidney mass. This surgical intervention triggers compensatory mechanisms in the remaining kidney to maintain a normal glomerular filtration rate (GFR) [1]. These adaptations include hypertrophy of the glomeruli and renal tubules, leading to an increase in single nephron GFR (SNGFR) [2]. However, prolonged hyperfiltration due to these compensatory mechanisms can contribute to kidney scarring and the development of hypertension [3].

Hypertension is a prevalent and significant comorbidity in patients with chronic kidney disease (CKD), affecting both adults and children [4]. Among the various etiologies of CKD, con-

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genital abnormalities of the kidney and urinary tract, including congenital solitary kidney, and acquired solitary kidney resulting from unilateral nephrectomy play a significant role. Approximately 60% of children with chronic kidney disease have congenital abnormalities of the kidney and urinary tract [5]. Additionally, glomerular causes, including acquired solitary kidney, account for 10-20% of pediatric CKD cases, with glomerular etiology representing as much as 45% of CKD cases in children over 12 years old in the United States [6].

In the congenital solitary kidney, resulting from a single intrauterine functioning kidney, compensatory hypertrophy begins intrauterine by increasing the number of nephrons, depending mainly on gestational age and placental blood flow [7]. As a result, the nephron number and size of the solitary kidney are expected to be greater than those of a single kidney in a normal newborn with both kidneys. In contrast, individuals undergoing unilateral nephrectomy after birth experience compensatory hypertrophy limited to the enlargement of existing nephrons without an increase in their number, predisposing them to a higher risk of glomerular injury [8].

Patients with congenital or acquired solitary kidney are classified as CKD stage 1 despite maintaining normal kidney function, according to the KIDIGO 2012 classification, due to the presence of structural renal abnormalities for more than 3 months [9]. After nephrectomy, the remaining kidney undergoes compensatory growth, increasing both kidney size and functional capacity to achieve similar glomerular solute clearance as individuals without nephrectomy. To achieve this with half the number of nephrons, early hyperfiltration is necessary [10, 11]. However, persistent hyperfiltration is a risk factor for progressive glomerular injury, leading to hypertension, proteinuria, and a permanent reduction in glomerular clearance, eventually progressing to end-stage renal disease (ESRD) [12].

In the clinical setting, proteinuria and hypertension are considered significant markers for the progression of CKD. The renin-angiotensin-aldosterone system (RAAS) is the main regulator of blood pressure. Some individuals do not achieve optimal blood pressure control including CKD patients, despite receiving maximum recommended doses of ACEi/ARBs [13]. In these cases, it becomes crucial to explore additional intrinsic renal pathways that may contribute to the development of hypertension in CKD patients.

Proteases, enzymes that break down proteins into smaller polypeptides, are being investigated as potential contributors to kidney pathophysiology. Among the proteases, renal KLK1 and cathepsins have emerged as key players in kidney function and blood pressure regulation [14, 15]. Renal KLK1, originally discovered in human urine, is expressed in tubular epithelial cells. It is involved in electrolyte and water homeostasis, blood pressure regulation, and inflammation [16]. Studies have shown that renal KLK1 deficiency is associated with sodium retention and increased blood pressure [17].

Furthermore, impaired renal excretion of KLK1 has been observed in hypertensive conditions, potentially leading to sodium accumulation and the development of hypertension [18]. Cathepsins are a group of proteases found in lysosomes [19], extracellular vesicles [20, 21], and biological fluids [22, 23]. These diverse groups of proteases play important roles in kidney physiology and pathology. Specifically, cathepsins B, D, L, and S have been implicated in the regulation of extracellular matrix homeostasis, autophagy, apoptosis, glomerular permeability, endothelial function, and inflammation [16]. Dysregulation of cathepsin expression and activity has been associated with hypertension development and kidney disease progression.

In this study, we investigated changes in protein expression and activity of KLK1 and multiple members of the cathepsin family of proteases in the kidney of hypertensive juvenile mice that underwent UNX or sham surgery. The understanding of the regulation of these proteases may contribute to the development of novel strategies for managing hypertension and preserving renal function in patients with CKD.

Methods

Animals

Four-week-old male C57B6 wild-type mice (Jackson laboratories; Bar Harbor, ME) were subject to sham surgery (SH) or left unilateral

nephrectomies (UNX). All animal procedures were performed under an approved University of Florida Institutional Animal Care and Use Committee (IACUC) protocol study #202011-157.

Animal diet and mouse metabolic cage studies

Each mouse was individually housed in a mouse metabolic cage at five weeks of age to allow for the calculation of water intake, urine output, and the collection of urine produced during the inactive and active phases. The mice from the two groups were maintained on a normal chow diet. Water intake and urine output were recorded daily. Urine was collected between 7-9 am representative of urine produced during the animal's active phase and between 7-9 pm representative of urine produced during the animal's inactive phase. The mice were euthanized by cervical dislocation at 9 weeks of age.

Blood pressure measurements

The IITC MRBP System (Life Science Inc.; Woodland Hills, CA, USA) was used to measure blood pressure twice a week during the animal's active phase (7 pm-9 pm) and inactive phase (7 am-9 am). Data were analyzed using Version 1.63 of the MRBP Software.

Preparation of kidney lysates

A 50 mg section of the kidney cortex from each SH and UNX mouse was homogenized using an Omni TH homogenizer (Warrenton, VA, USA) in 500 mL of tissue protein extraction reagent (TPER) (ThermoFisher Scientific). The tissue lysates were solubilized on ice for 20 minutes while vortexing the samples every 5 minutes, and then centrifuged using a Micromax benchtop centrifuge (Thermo IEC) at 13,000 rpm for 10 minutes at room temperature. The supernatant was transferred to clean vials and then subjected to ultracentrifugation using an optima L-90K ultracentrifuge (Beckman Coulter; Schaumburg, IL, USA) and SW55 rotor (Beckman Coulter) at 34,000 rpm for 30 minutes at 4°C. The soluble fraction (supernatant) was collected in separate tubes. Next, 200 µl of TPER was used to resuspend the pellets, followed by sonication on ice for two 3-second intervals, and the resulting membrane fraction was transferred to clean tubes.

BCA protein assay

The total protein concentration from each tissue lysate was calculated after performing a bicinchoninic acid protein assay (BCA) protein assay (ThermoFisher Scientific). First, a 1:10 dilution of the soluble or membrane kidney cortex protein lysate was prepared in ddH_2O. Nine standards were prepared from serial dilutions from a stock 2 mg/ml concentration of BSA (ThermoFisher Scientific) and the protein concentrations of each sample were determined from the equation corresponding to the curve generated from the standards.

SDS-PAGE and Western blotting

A total of 50 µg of protein from soluble or membrane fraction kidney tissue lysates were resolved on 4-20% Tris HCl polyacrylamide gels using the Criterion electrophoresis system (Bio-Rad; Hercules, CA). The proteins were transferred in Towbin buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol) onto nitrocellulose membranes (ThermoFisher Scientific) using the Criterion transfer system (Bio-Rad). The membranes were then blocked in 5% nonfat dry milk in 1× Tris-buffered saline (Bio-Rad) for 1 hour at room temperature and then washed three times with 1× TBS before being incubated with a 1:1000 dilution of primary antibody (**Table 1**) on a rocker for at least 8 hours at 4°C. Next, the membranes were washed three times with 1× TBS and incubated with a 1:3000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (BioRad) prepared in blocking solution for 1 hour at room temperature. Next, the membranes were washed three times with 1× TBS, incubated with ECL reagent (BioRad) for 7 minutes, and then imaged using a Bio-Rad imager.

Nitric oxide assay

The nitric oxide (NO) concentration was determined in urine samples using a NO assay kit (**Table 2**) according to the manufacturer's instructions.

Cathepsin assays

The activities of Cathepsin B, S, and D were measured in the tissue lysates using cathepsin assay kits (Abcam) (**Table 2**) according to the manufacturer's instructions.

Table 1. Antibodies used in this study

Antibody	Cat No.	Manufacturer	Application
Cathepsin B	31718	Cell Signaling Technologies	WB/IHC
Cathepsin S	ab232740	Abcam	WB
Cathepsin D	69854	Cell Signaling Technologies	WB
KLK1	PA1709	BOSTER	WB/IHC
Cathepsin S	Sc-271619	Santa Cruz Biotechnology	IHC
Cathepsin D	Sc-377299	Santa Cruz Biotechnology	IHC
Bradykinin B1 R	Sc-518136	Santa Cruz Biotechnology	IHC

WB refers to Western blot. IHC refers to immunohistochemistry.

Table 2. Assays used in this study

Assay	Cat No.	Manufacturer
Cathepsin B	Ab65300	Abcam
Cathepsin S	Ab65307	Abcam
Cathepsin D	Ab65302	Abcam
Furin	78040	BPS Bioscience
Nitric oxide	Ab272517	Abcam
Creatinine	Ab204537	Abcam
Cystatin C	EMCST3	Invitrogen
Plasma renin	EMREN1	Invitrogen
Atrial natriuretic peptide	Ab108797	Abcam

Plasma ANP measurements

Plasma ANP was measured using an ANP ELISA kit (**Table 2**) while following the manufactures instructions.

Serum creatinine measurements

Serum creatinine concentrations were determined using a creatinine assay kit (**Table 2**) according to the manufacture's instructions.

Serum cystatin C measurements

Serum cystatin C concentrations were determined using a cystatin C assay kit (**Table 2**) according to the manufacturer's instructions using a 1:400 sample dilution.

Plasma renin measurements

Plasma renin concentrations were measured using a Mouse Renin 1 ELISA kit (**Table 2**) according to the manufacturer's instructions using a 1:20 sample dilution.

Statistical analyses

Prism 9 software (San Diego, CA) was used to analyze each data set. An independent variable between the two study groups was compared using an unpaired two-tailed t-test, while an independent variable between multiple groups was compared using a one-way ANOVA. A Tukey post hoc test was performed. For two variables between multiple groups, a two-way ANOVA was performed. Data are presented as mean ± standard deviation. A p-value < 0.05 was defined as being statistically significant.

Results

Blood pressure differences during the active and inactive phases of juvenile mice with UNX or SH surgery

Since blood pressure exhibits a circadian rhythm and shows differences over a 24 phase, we investigated changes in blood pressure in male C57B6 wild-type mice subject to UNX or SH surgery during the active and inactive phases. During the active phase, the systolic blood pressure in the UNX group exhibited a significant increase compared to the SH control group (**Figure 1A**). This elevation in blood pressure was also observed during the inactive phase (**Figure 1B**), and the difference was persistent and progressive from week 3 to week 5 post-procedure. Additionally, when comparing blood pressure within the UNX group during both active and inactive phases, a significant increase was found during the active phase compared to the inactive phase (**Figure 1C**).

Serum creatinine and cystatin C levels in juvenile mice with UNX or sham surgery

Measurement of serum creatinine, a marker of glomerular filtration rate, at week 2 and week 4 post-procedure during the active and inactive phases showed no significant difference between the UNX and SH groups (**Figure 2A**). Similarly, levels of cystatin C, another marker for kidney function, at week 3 and week 5 post-procedure during the active and inactive phases did not differ significantly between the two groups (**Figure 2B**).

Plasma renin activity levels in juvenile mice with UNX or sham surgery

Since overactivation of the renin-angiotensin-aldosterone system (RAAS) can lead to the development of hypertension, we investigated

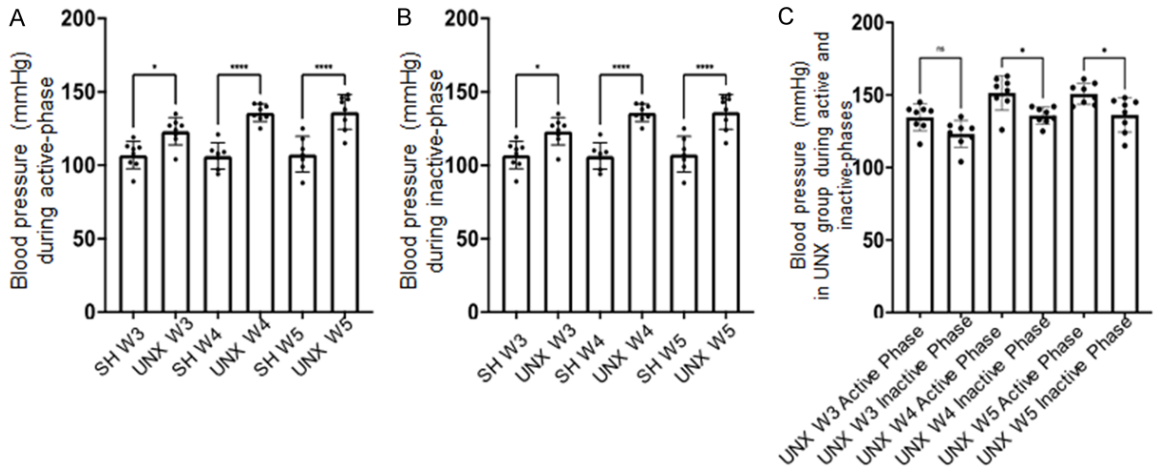

Figure 1. Changes in blood pressure in sham (SH) and unilateral nephrectomized (UNX) mice during the active and inactive phases. A. Tail cuff systolic blood pressure measurements during the active phases of SH and UNX mice. B. Tail cuff systolic blood pressure measurements during the inactive phase of SH and UNX mice. C. Tail cuff systolic blood pressure measurements in the UNX group during the active and inactive phases. n=8 mice per group. The results were analyzed using a student t-test to compare differences between the two groups. *P < 0.1, **P < 0.01, ****P < 0.0001.

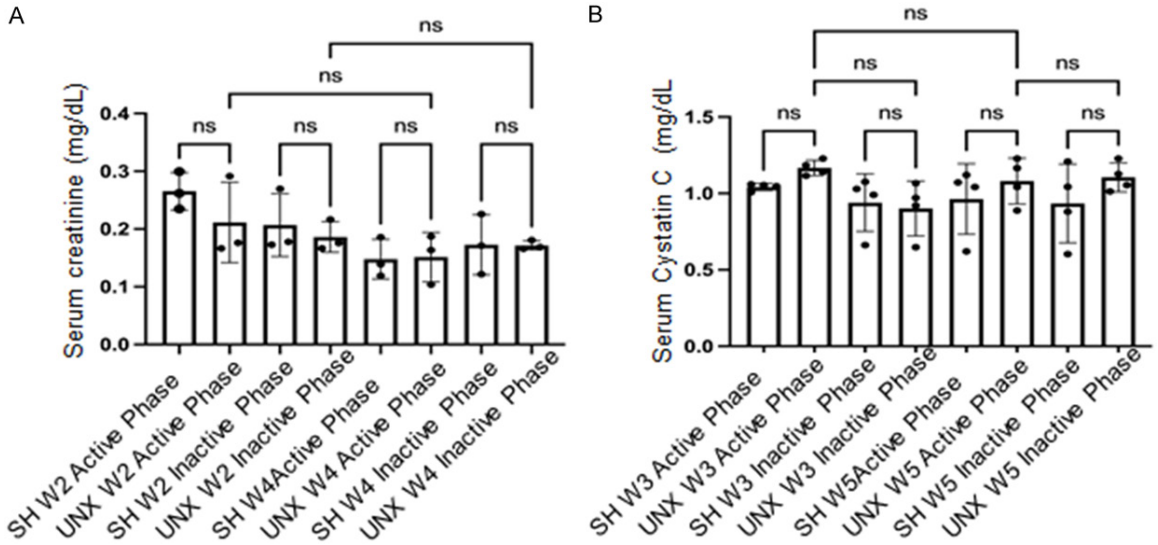

Figure 2. Measurement of the kidney function markers in sham (SH) and unilateral nephrectomized (UNX) juvenile mice during the active and inactive phases. A. Serum creatinine levels in week 2 & week 4 post-procedure urine samples from the active and inactive phases. N=3 samples per group. B. Serum cystatin C levels in week 3 & week 5 post-procedure urine samples from the active and inactive phases. The results were analyzed using a two-way ANOVA to compare differences between the two groups and between different time points. ns stands for not statistically significant. N=4 samples per group.

whether circulating renin levels differ between juvenile mice that underwent UNX or SH surgery. There was no significant difference in plasma renin activity between the UNX and SH groups at week 1 and week 3 post-procedure during both the active and inactive phases (**Figure 3**).

Plasma atrial natriuretic peptide and natriuretic peptide receptor C levels in UNX and sham mice

Since atrial natriuretic peptide (ANP) plays a seminal role in blood pressure homeostasis, we measured its levels from the plasma of UNX

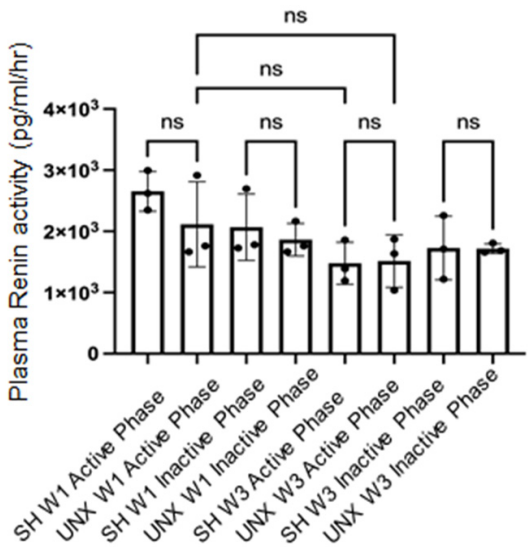

Figure 3. Measurement of plasma renin in sham (SH) and unilateral nephrectomized (UNX) mice. Plasma renin was measured in week 1 and week 3 post-procedure plasma samples from the active and inactive phases. The results were analyzed using a two-way ANOVA to compare differences between the two groups and between different time points. ns stands for not statistically significant. N=3 samples per group.

and SH mice. In addition, we measured protein expression of the c-type natriuretic peptide receptor (NPRC), one of the main proteins responsible for its clearance during the animal's inactive and active phases. Measurement of plasma ANP levels at week 2 and week 4 post-procedure during both the active and inactive phases revealed no significant differences between juvenile mice with UNX and SH surgery (**Figure 4A**). Additionally, Western Blot analysis of NPRC showed no appreciable difference in protein expression between the two groups (**Figure 4B-D**).

Western blot and densitometric analysis of KLK1 and cathepsins in the kidney cortex of UNX or sham mice

Since a myriad of proteases have been shown to play a role in blood pressure regulation, we investigated whether there are changes in some of the main proteases expressed in the kidney of juvenile mice that underwent UNX or SH surgery. Western Blot and densitometric analysis of KLK1 in kidney cortex lysates showed a decrease in KLK1 protein expression in the UNX group compared to the SH control group (**Figure 5A**). In contrast, cathepsin B, cathepsin D, and cathepsin S protein expressions were significantly upregulated in the UNX group compared to the SH control group (**Figure 5B-D**).

Immunohistochemistry analysis of KLK1 and cathepsins in UNX and sham mice

To corroborate the Western blot and densitometric analyses of KLK1 and various cathepsins, we performed immunohistochemistry for KLK1 and cathepsin B, D, and S. IHC analysis confirmed down-regulation of KLK1 protein expression (**Figure 6A**) and upregulation of cathepsin B, cathepsin D, and cathepsin S (**Figure 6B-D**) protein expressions in the kidneys of the UNX group compared to the SH control group.

Urinary excretion of cathepsins

As shown in **Figure 7A**, urinary cathepsin B activity levels were significantly lower in the juvenile mice that underwent UNX compared to SH control mice. Although cathepsin S levels were not statistically significant between the two groups, there was a lower trend for urinary cathepsin S activity in the UNX group compared to the SH control group (**Figure 7B**).

Bradykinin receptors expression in UNX and sham mice

Next, we investigated whether there were changes in the protein expression of bradykinin receptor B1 since bradykinin receptors are known to play a role in blood pressure regulation. Immunohistochemistry of kidney tissue from UNX and SH mice showed a significant down-regulation of bradykinin B1 receptor protein expression in the UNX group compared to the SH control group (**Figure 8**).

Urinary nitric oxide levels in UNX and sham mice

Since the bradykinin-NO pathway has been implicated in blood pressure regulation, we next investigated whether there were differences in mice that underwent UNX compared to SH surgery. Measurement of NO urinary excretion during the active phase demonstrated a

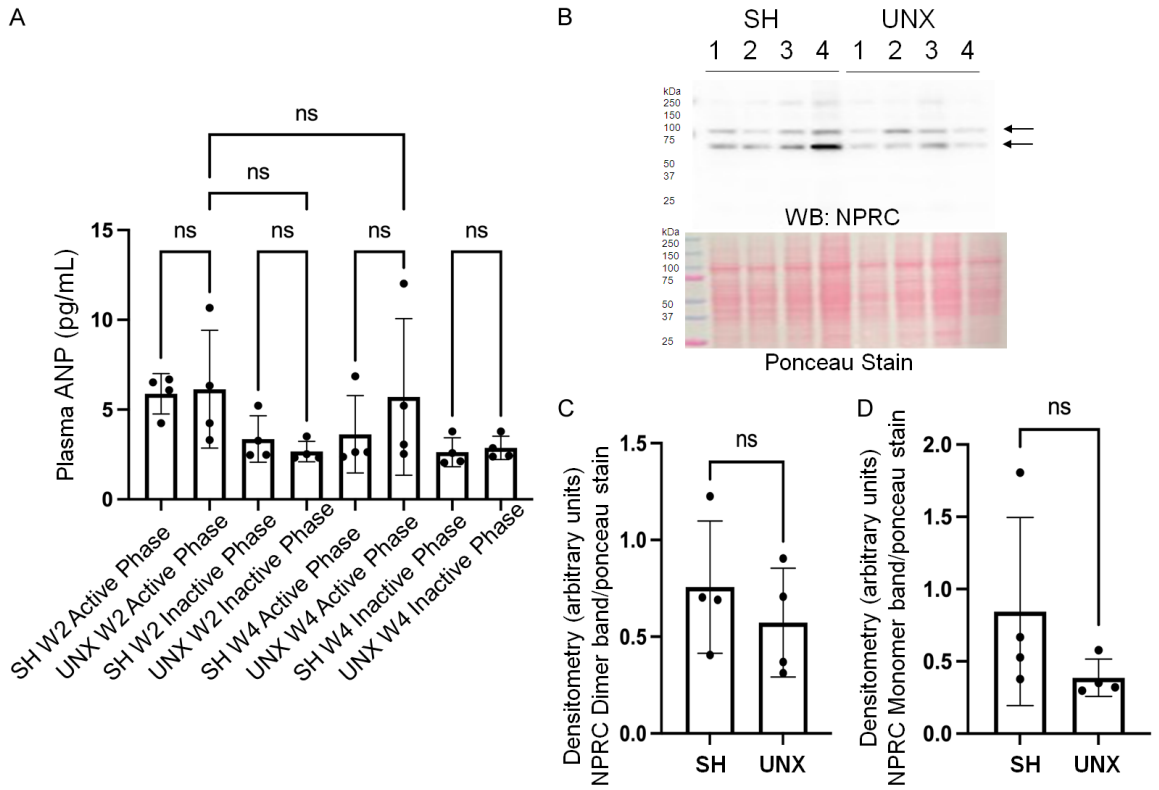

Figure 4. Measurement of plasma atrial natriuretic peptide (ANP) and natriuretic peptide receptor C (NPRC) protein expression in kidney lysates from sham (SH) and unilateral nephrectomized (UNX) mice. A. Measurement of ANP levels in week 2 and week 4 post-procedure plasma samples from the active and inactive phases. N=4 samples per group. B. Western blot of natriuretic peptide receptor C (NPRC) protein expression in kidney cortex lysates from SH and UNX mice. C. Densitometric analysis of the immunoreactive band corresponding to the NPRC dimer. D. Densitometric analysis of the immunoreactive band corresponding to NPRC monomer. The results were analyzed using a two-way ANOVA to compare differences between the two groups and between different time points. ns stands for not statistically significant. N=4 samples per group.

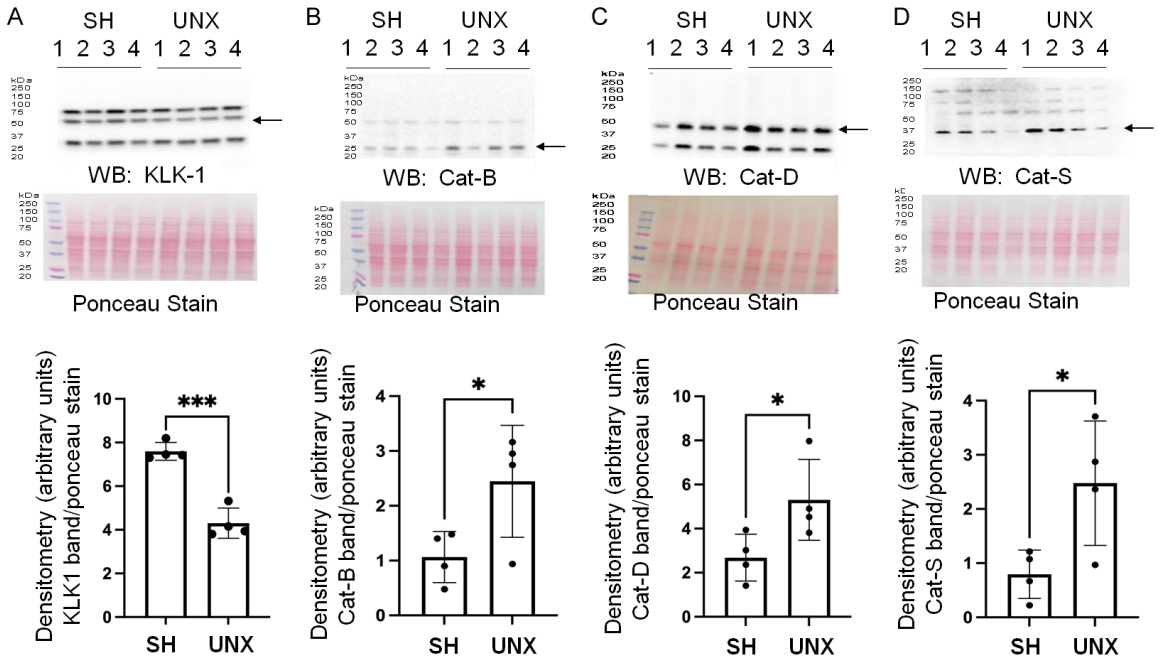

Figure 5. Western blot and densitometric analysis of various proteases in kidney lysates from sham (SH) and unilateral nephrectomized (UNX) mice. A. Western blot (top) of kidney kallikrein 1 (KLK1) protein expression in kidney lysates from SH and UNX mice. Densitometric analysis (bottom) of the immunoreactive band corresponding to KLK1. B. Western blot (top) of cathepsin B (Cat-B) protein expression in kidney lysates from SH and UNX mice. Densitometric analysis (bottom) of the immunoreactive band corresponding to cathepsin B. C. Western blot (top) of cathepsin D (Cat-D) protein expression in kidney lysates from SH and UNX mice. Densitometric analysis (bottom) of the immunoreactive band corresponding to cathepsin D. D. Western blot (top) of cathepsin S (Cat-S) protein expression in kidney lysates from SH and UNX mice. Densitometric analysis (bottom) of the immunoreactive band corresponding to cathepsin S. *P < 0.05. N=4 mice per group. A Student t-test was used to compare the differences between the two groups. N=4 samples per group.

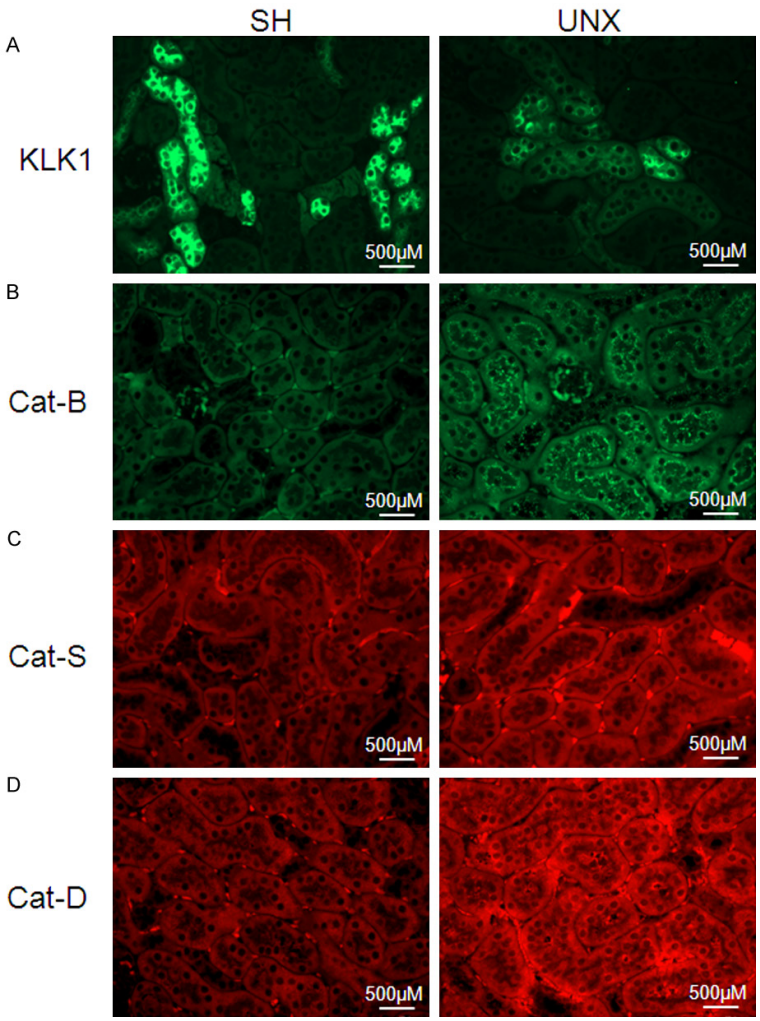

Figure 6. Immunohistochemistry (IHC) of KLK1 and Cathepsin isoform expression in the kidney from sham (SH) and unilateral nephrectomized (UNX) mice. A. IHC of kidney kallikrein 1 (KLK1) protein expression in kidney lysates from SH and UNX mice. B. IHC of kidney cathepsin B (Cat-B) protein expression in kidney lysates from SH and UNX mice. C. IHC of kidney cathepsin S (Cat-S) protein expression in kidney lysates from SH and UNX mice. D. IHC of kidney cathepsin D (Cat-D) protein expression in kidney lysates from SH and UNX mice. 40X magnification. N=4 mice per group. The scale bar denotes 500 μM. Images are at 40X magnification.

significant decrease in the UNX group compared to the sham control group (**Figure 9**).

Discussion

Unilateral nephrectomy (UNX) is a surgical procedure commonly performed in cases of renal diseases or organ donation, resulting in the removal of one kidney. The remaining kidney undergoes compensatory mechanisms to maintain a normal glomerular filtration rate (GFR) [1]. However, these adaptations can potentially lead to long-term complications such as hypertension and kidney damage. Although various proteases have been shown to cleave and activate proteins involved in blood pressure regulation, their regulation during compensatory hypertrophy is largely unknown.

Here we investigated changes in blood pressure in juvenile mice that were subject to UNX, compared to sham surgery in a time-of-day dependent manner, since blood pressure follows circadian rhythms that can be disrupted in pathophysiology. Blood pressure is typically higher in the active phase compared to the inactive phase within a 24-hour period [24]. Also, studies have shown that patients that lack the normal nocturnal blood pressure fall or those with an excessive morning blood pressure surge are at risk for adverse cardiovascular outcomes [25]. Our data showed a sustained elevation in systolic blood pressure in the UNX group compared to

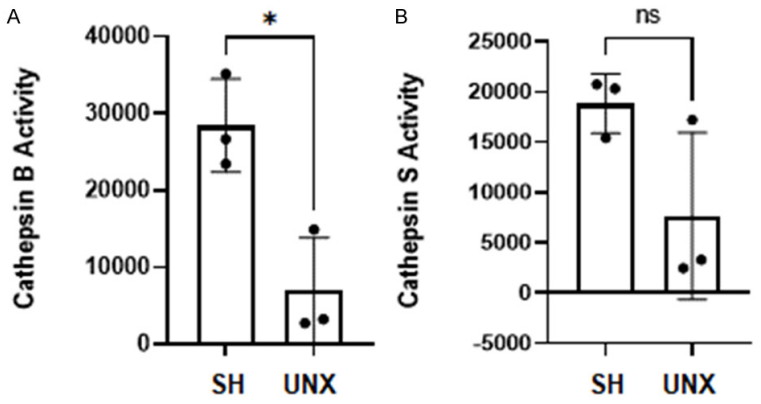

Figure 7. Urinary excretion of cathepsin B and cathepsin S from sham (SH) and unilateral nephrectomized (UNX) mice. A. Cathepsin B activity assay showing relative activity of the protease in urine samples collected 3 weeks after SH or UNX. N=3 samples per group. B. Cathepsin S activity assay showing relative activity of the protease in urine samples collected 3 weeks after SH or UNX. A Student's t-test was used to compare the differences between the two groups. N=3 samples per group. * represents a *p*-value < 0.05. ns stands for not statistically significant.

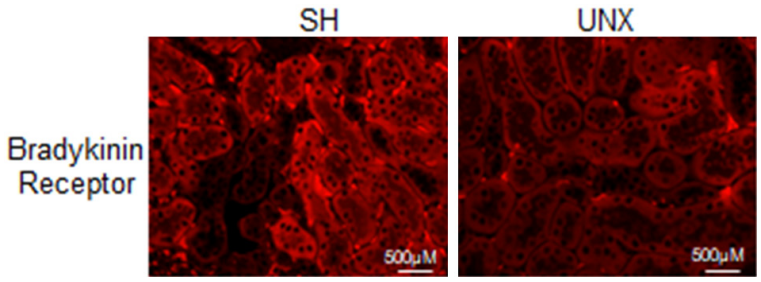

Figure 8. Immunohistochemistry (IHC) of Bradykinin receptor B1 expression in the kidney from sham (SH) and unilateral nephrectomized (UNX) mice. IHC of Bradykinin B1 receptor expression in the kidney from SH and UNX mice. 40X magnification. N=4 mice per group. The scale bar denotes 500 μM. Images are at 40X magnification.

the sham-operated control group during both the animal's active and inactive phases. This finding suggests the development of hypertension following UNX. Our results are consistent with the results from a study by Ohashi et al which showed that night-to-day ratios of systolic blood pressure were elevated after nephrectomy [26]. One of the significant observations in our study was the sustained elevation in systolic blood pressure in the UNX group compared to the sham-operated (SH) control group during both the active and inactive phases. This finding suggests the development of hypertension following UNX and that aligned with Ohash et al- 2016 [26]. Furthermore, the higher blood pressure during the active phase, along with preserved night dipping and the circadian rhythm with diurnal variations in blood pressure in UNX group indicates that UNX did not immediately impact kidney function, as evidenced by the lack of significant differences in serum creatinine and Cystatin C levels between the UNX and SH groups in our study. These results confirm the hypnosis proved by Fukuda et al-2006, for the inverse relationship between GFR and the night/day ratio of BP [27]. However, it is essential to conduct longer-term studies to evaluate potential changes in kidney function over time. Additionally, the absence of a circadian rhythm in blood pressure has been observed in patients with chronic kidney disease undergoing hemodialysis due to the complete loss of residual renal function [28]. These findings highlight the potential role of residual kidney function in maintaining the circadian rhythm of blood pressure.

The heart and kidneys play crucial roles in regulating salt and water balance within the body. A key player in this regulation is the cardiac hormone ANP. This hormone promotes salt excretion, reduces blood volume, and induces vessel relaxation, thereby maintaining electrolyte and fluid balance [29, 30]. The physiological significance of the ANP pathway has been demonstrated in various animal studies. For instance, mice lacking ANP have been shown to develop hypertension, while ANP overexpression leads to hypotension [31]. In our study, we aimed to examine changes in ANP and its clearance receptor, NPRC after unilateral UNX or SH. Interestingly, we found no significant differences in plasma ANP levels or differences in protein expression of NPRC between the UNX and sham control mice. These results suggest that UNX may not directly influence the release of ANP or alter the expression of NPRC in the kidney. There are some important differences between our study and other studies that could explain opposing results for ANP. First, we

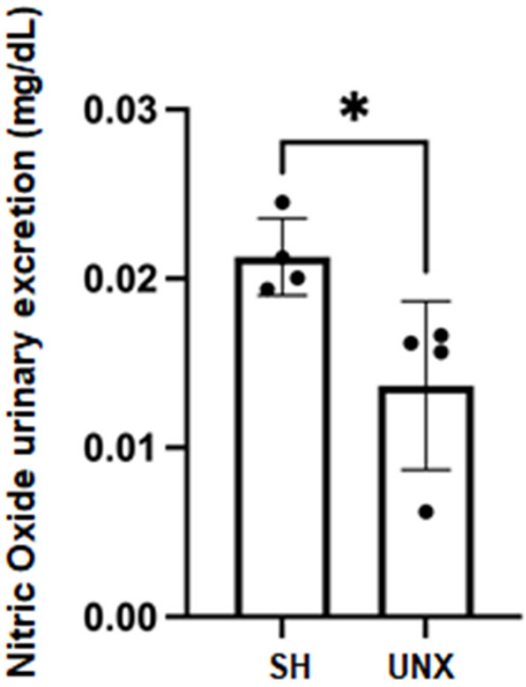

Figure 9. Nitric Oxide urinary excretion in sham (SH) and unilateral nephrectomized (UNX) mice. The results were analyzed using a Student's t-test to compare differences between the two groups. * represents a *p*-value < 0.05. N=4 samples per group.

focused on specific time points following UNX, but it is possible that changes in ANP levels and NPRC protein expression may occur at different time points. Second, our study was conducted in a juvenile mice model and the physiological response to UNX and the regulation of the natriuretic peptide system may differ between juvenile mice and adult mice.

The renin-angiotensin-aldosterone system (RAAS) has long been recognized as a key regulator of blood pressure [32], including patients with CKD [33]. In our study, we aimed to investigate the role of RAAS in the development of hypertension following UNX in the presence of normal kidney function and plasma renin activity. Initially, we measured plasma renin activity in the UNX and sham groups at week 1 and week 3 post-procedure, during both the active and inactive phases. Surprisingly, we did not observe a significant difference in plasma renin activity between the two groups. This finding suggests that UNX may not directly influence the rate-limiting enzyme in the RAAS pathway.

Renal KLK1 and cathepsin proteases have emerged as key players in kidney function and blood pressure regulation [34]. In our study, we investigated the role of these proteases in the development of hypertension following UNX. We observed a significant down-regulation of KLK1 protein expression in the UNX group compared to the sham control group. KLK1 is known to play a crucial role in the regulation of renal hemodynamics and blood pressure through the activation of the kallikrein-kinin system [34]. Furthermore, the kallikrein-kinin system is thought to interact with RAAS to regulate water-electrolyte handing and blood pressure regulation [34]. KLK1 exerts its effects by inducing the release of bradykinin, a potent vasodilator [18]. Decreased renal bradykinin generation has been associated with sodium accumulation and the development of hypertension [35-37]. In our study, we observed a significant down-regulation of the bradykinin B1 receptor protein expression in the UNX group compared to the SH control group. The observed down-regulation of the B1 receptor further suggests a disruption in this regulatory pathway following UNX. Several studies have demonstrated the beneficial effects of KLK1 in improving renal function. KLK1 has been shown to increase glomerular filtration rate, renal blood flow, and NO production [38-42]. NO is a potent vasodilator and plays a critical role in maintaining renal and cardiovascular homeostasis [49]. The reduced NO excretion observed in the UNX group of our study indicates impaired NO production or availability, which may contribute to the development of hypertension and renal dysfunction. KLK1 also exhibits anti-inflammatory, anti-oxidative, anti-fibrotic, and anti-apoptotic actions in animal models of renal injury [38-42]. Inhibition of endogenous KLK1 activity has been found to increase inflammatory cell infiltration, myofibroblast, and collagen deposition in kidneys, indicating the protective role of KLK1 in renal inflammation and fibrosis [43].

The upregulation of cathepsin B, cathepsin D, and cathepsin S protein expressions observed in the UNX group compared to the SH control group highlights the potential involvement of these proteases in renal adaptations and tissue remodeling. Cathepsins, known for their proteolytic activity, play crucial roles in various physiological and pathological processes, including renal remodeling and fibrosis [16]. Cathepsin B, a cysteine protease, has been shown to activate prorenin to renin [44], in-

crease alpha ENaC channel activity, and subsequently lead to the development of hypertension [45]. This suggests that the upregulation of cathepsin B in the UNX group may contribute to enhanced sodium reabsorption and the development of hypertension. Moreover, dysregulation of cathepsin B expression/activity has been linked to inflammatory processes and the release of cytokines [16]. Cathepsin D, an aspartic endopeptidase primarily found in lysosomes, has traditionally been associated with non-specific protein degradation in the acidic environment of lysosomes. However, emerging evidence suggests its regulatory role in apoptosis and inflammation along with its involvement in various pathological processes such as atherosclerosis [46]. Additionally, cathepsin D is a risk factor for endothelial dysfunction in chronic kidney disease, independent of traditional cardiovascular risk factors [47]. Cathepsin S was shown to co-localize with integrin 3 on the surface of vascular smooth muscle cells and contribute to elastolytic activity and invasive behavior of smooth muscle cells [48]. Its activation, liberation, and modification of angiogenic growth factors, cytokines, and proteases contribute to lipid metabolism degradation, and alterations of various cellular functions including migration, invasion, proliferation, apoptosis, angiogenesis, and matrix protein remodeling [16]. Increased levels of cathepsin S have been associated with cardiovascular disease (CVD) and may serve as an early biomarker for CVD in CKD [16]. The dysregulation of cathepsins B, D, and S in the UNX group suggests their potential roles as key players in kidney pathophysiology, the development of hypertension, and the progression of kidney disease. Understanding the precise mechanisms by which these cathepsins contribute to renal adaptations, tissue remodeling, hypertension, atherosclerosis, and endothelial dysfunction in CKD will be crucial for developing targeted therapeutic interventions.

Collectively, our findings highlight the complex interplay of multiple factors following UNX, including alterations in blood pressure regulation, protein expression, receptor expression, and NO urinary excretion. These observations provide valuable insights into the potential mechanisms underlying the development of hypertension and renal dysfunction in the context of UNX. Further studies are warranted to elucidate the molecular pathways involved and to explore potential therapeutic interventions targeting these pathways to mitigate the long-term complications associated with UNX.

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Disclosure of conflict of interest

None.

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