Role of the Primary Cilia on the Macula Densa and Thick Ascending Limbs in Regulation of Sodium Excretion and Hemodynamics

Jiangping Song, Lei Wang, Fan Fan, Jin Wei, Jie Zhang, Yan Lu, Yiling Fu, Shaohui Wang, Luis A. Juncos, Ruisheng Liu

Abstract—We investigated the significance of the primary cilia on the macula densa and thick ascending limb (TAL) in regulation of renal hemodynamics, sodium excretion, and blood pressure in this study. A tissue-specific primary cilia knock-out (KO) mouse line was generated by crossing NKCC2-Cre mice with IFT88-A/flox mice (NKCC2Cre; IFT88AΔflox), in which the primary cilia were deleted from the macula densa and TAL. NO generation was measured with a fluorescent dye (4,5-diaminofluorescein diacetate) in isolated perfused juxtaglomerular apparatus. Deletion of the cilia reduced NO production by 56% and 42% in the macula densa and TAL, respectively. NO generation by the macula densa was inhibited by both a nonselective and a selective nitric oxide synthesis inhibitors, whereas TAL-produced NO was inhibited by a nonselective and not by a selective NO synthesis 1 inhibitor. The tubuloglomerular feedback response was enhanced in the KO mice both in vitro measured with isolated perfused juxtaglomerular apparatuses and in vivo measured with micropuncture. In response to an acute volume expansion, the KO mice exhibited limited glomerular filtration rate elevation and impaired sodium excretion compared with the wild-type mice. The mean arterial pressure measured with telemetry was the same for wild-type and KO mice fed a normal salt diet. After a high salt diet, the mean arterial pressure increased by 17.4±1.6 mm Hg in the KO mice. On the basis of these findings, we concluded that the primary cilia on the macula densa and TAL play an essential role in the control of sodium excretion and blood pressure. (Hypertension. 2017;70:324-333. DOI: 10.1161/HYPERTENSIONAHA.117.09584.) • Online Data Supplement

Key Words: blood pressure • cilia • diet • kidney • mice

Primary cilia dysfunctions, collectively termed as ciliopathies, have been linked to numerous human diseases and genetic disorders and present with a broad range of clinical features, including polycystic kidney disease, retinal degeneration, and brain malformations.1–6 Primary cilia are nonmotile sensory antennae, extending from the surface of many eukaryotic cells, including the macula densa cells.7–12 Although the function of the primary cilia in most cells has largely remained elusive, they have been known to serve as mechanosensors in the mammalian kidney and vascular endothelial cells.1,3,5,33 We recently demonstrated that shear stress stimulates the primary cilia on the macula densa, enhancing its NO generation and thereby inhibiting the tubuloglomerular feedback (TGF) in acute experiments in vitro.32 However, the long-term physiological consequences of this mechanism are not known.

Increase of tubular flow raises NaCl delivery to the macula densa promoting the release of adenosine and ATP, which constricts the afferent arteriole and decreases single nephron glomerular filtration rate (GFR), a process that is called the TGF response. NO generated in the macula densa by neuronal nitric oxide synthesis (NOS1), the predominant isoform expressed in macula densa cells,14,15 inhibits the TGF response.16–19 We recently generated a tissue-specific NOS1 deletion mouse line, in which NOS1 was specifically deleted from the macula densa.20 These mice developed salt-sensitive hypertension, associated with enhanced TGF responsiveness, attenuated increases in GFR, and impaired sodium excretion.20 These studies demonstrated the physiological significance of macula densa–derived NO and TGF responsiveness in the long-term control of hemodynamics. However, the importance of the primary cilia on the macula densa in the long-term regulation of renal hemodynamics, sodium excretion, and blood pressure has not been investigated. In this study, we tested the hypothesis that the primary cilia on the macula densa blunt the
TGF response by enhancing NO generation, which promotes GFR elevation, increases sodium excretion, and contributes to maintaining the electrolyte and volume hemostasis.

**Methods**

All procedures and experiments were approved by the Institutional Animal Care and Use Committee at the University of South Florida College of Medicine and the University of Mississippi Medical Center. All chemicals were purchased from Sigma (St. Louis, MO) except as indicated. Male mice at age of 8 to 12 weeks were used. Littermate age-matched wild-type (WT) mice with C57BL6/6 background were used as control for the knock-out (KO) mice.

**Microperfusion**

The afferent arteriole and attached macula densa were isolated and micropерfused as described previously and described in online-only Data Supplement.

**Immunofluorescence**

Similar methods were used as we previously reported and described in the online-only Data Supplement.

**Identification of Primary Cilia on Macula Densa Cells in the Isolated Perfused Juxtaglomerular Apparatus**

We detected primary cilium on the macula densa with immunofluorescence in the isolated perfused rabbit juxtaglomerular apparatus (JGA) as we previously reported and described in online-only Data Supplement.

**Measurement of NO in Isolated Perfused JGA**

We measured NO production in the macula densa and the thick ascending limb (TAL) using a cell permeable fluorescent NO indicator 4-amino-5-methylamino-2′,7′-difuoro fluorescein diacetate as previously reported and described in online-only Data Supplement.

**Isolation of Macula Densa Cells**

Laser capture microdissection was used to isolate macula densa cells from frozen kidney slices, as we previously reported and described in online-only Data Supplement.

**Real-Time PCR and Western Blot to Measure Splice Variants of NOS1**

Real-time PCR and Western blot measurement are described in the online-only Data Supplement.

**Measurement of [Ca^{2+}] in Isolated Perfused JGAs and Cultured MMDD1 Cells**

**Intracellular Calcium Measurement**

Similar methods were as we previously reported and described in the online-only Data Supplement.

**Shear Stress Adjustment in Isolated Perfused JGAs**

The viscosity of the tubular perfusate was increased by adding a high molecular weight dextran (molecular weight: 200000; MP Biomedicals, Solon, OH) to a perfusate containing 80 mmol/L NaCl solution as we reported previously and described in the online-only Data Supplement.

**MMDD1 Cells**

Similar methods were used for MMDD1 cell culture, shear stress adjustment, small interfering RNA (siRNA) treatment, and [Ca^{2+}] measurement as we previously reported and described in the online-only Data Supplement.

**GFR Measurement in Conscious Mice**

We used a single bolus injection of fluorescein isothiocyanate (FITC)-inulin, similar to a previously published method for measurement of GFR in conscious mice, which is described in the online-only Data Supplement.

**Renal Clearance in Response to Isotonic Volume Expansion**

Methods used for measurement of kidney clearance function were similar to those that we recently reported and described in the online-only Data Supplement.

**Micropuncture**

Methods for animal preparation were the same as we previously published and described in the online-only Data Supplement.

**Telemetry Transmitter Implantation**

Similar methods for transmitter implantation and mean arterial pressure (MAP) monitoring were used as we described previously and described in the online-only Data Supplement.

**Results**

**Development of a Tissue-Specific Primary Cilia Knockout Mouse Strain**

We recently developed an NKCC2-cre mouse line. By crossing the NKCC2-Cre mice with IFT88Δflox/flox mice, we generated a tissue-specific cilia deletion mouse line (NKCC2ΔCre; IFT88Δflox, hereafter referred to as KO). The KO mice were normal in activity and development. No apparent cysts were observed in the kidney slices under light microscopy in the mice used for this study at ages of 10 to 14 weeks.

To characterize the KO strain and determine whether the primary cilia were deleted from the macula densa and TAL, we labeled the kidney slices with a primary antibody against acetylated α-tubulin for primary cilia. NKCC2 was clearly visualized in the apical membrane of the macula densa cells and the TAL in the renal cortex (Figure 1Aa) and medulla (Figure 1Ab) in both WT and KO mice. In the renal cortex, the primary cilium were present in the lumen of the TAL (Figure 1Ac) and medulla (Figure 1Ad) in both WT and KO mice. No apparent cysts were present in the KO mice (Figure 1Aa), whereas they were absent in the KO mice (Figure 1Ad). To show the primary cilia on the macula densa, we captured immunofluorescent imaging in the isolated perfused JGAs. Figure 1Ba shows representative images of the perfused macula densa and the TAL where the primary cilia were visualized in the WT mice indicated by white arrows, but were absent in the KO mice (Figure 1Bb). The left side reveals light microscopic images of the same perfused JGAs, indicating their anatomic structure. The orange arrow indicates a cilium on the TAL in a WT mouse in Figure 1Ba. The red arrows indicate cilium in glomeruli WT and KO mice. Over 90% of the primary cilia were deleted from the macula densa and TAL in KO mice compared with the WT mice (Figure 1C).

**Role of the Primary Cilia in NO Generation by the Macula Densa and TAL**

We next compared NO generation with 4,5-diaminofluorescein diacetate in isolated perfused JGAs from the KO and WT mice. We perfused the distal tubule of the JGA and measured the NO generation by the macula densa as indicated by red arrows in Figure 2A. We perfused the TAL of the JGA and
measured NO production by the TAL as noted by yellow arrows in Figure 2A.

The NO generation by the macula densa was 103±8.7 U/min in the WT mice, and it decreased to 45±3.4 U/min in the KO mice. The NO generation by the TAL was 21±2.6 U/min in the WT mice, and it decreased to 11±1.8 U/min in the KO mice (Figure 2B).

To determine the source of NO produced by the macula densa and TAL, we used the nonselective NOS inhibitor, L-N^G-nitroarginine methyl ester (L-NAME), and a selective NOS1 inhibitor, 7-nitroindazole (7-NI). Whereas L-NAME inhibited NO generation from both the macula densa and the TAL, 7-NI inhibited NO generation by the macula densa, but had no effect on NO generation by the TAL in both WT and KO mice (Figure 2C).

**Effect of High Salt Intake on NOS1 Splice Variant Expressions in KO Mice**

To determine if deletion of the primary cilia affect the expression levels of NOS1 splice variants in response to a high salt diet, we fed the KO mice with a high salt diet containing 4% NaCl for 7 days and measured the mRNA and protein levels of NOS1 splice variant. The KO and WT mice fed with normal salt diet were used as controls.

Deletion of primary cilia reduced macula densa NOS1β by about 30% in mRNA and protein levels compared between WT and KO mice fed normal salt diet (P<0.05; Figure 2D and 2E). NOS1β expression increased by >2-folds in mRNA (from 6.1±0.5 to 13.7±1.1 U) and protein (from 3.5±0.2 to 7.1±0.7 U) levels in the KO mice fed a high salt diet versus a normal salt diet.
diet ($P<0.01$, Figure 2D through 2F). The expression levels of NOS1α were no significant changes in KO and WT mice.

**Role of Shear Stress in Intracellular Calcium In Vitro**

**Isolated Perfused JGAs**

To test the effect of shear stress on intracellular calcium concentration ([Ca$^{2+}$]), we increased viscosity by adding dextran to the tubular perfusate while maintaining a constant tubular perfusion at 40 nL/min. Shear stress of tubular perfusate was increased from 0.81±0.04 to 1.57±0.07 mPa s by adding dextran in 80 mmol/L NaCl macula densa solution.

When we increase viscosity from low to high, [Ca$^{2+}$] increased from 89±7.9 to 142±11.3 nmol/L in the macula densa and from 95±8.1 to 126±12.5 nmol/L in the TAL in the WT mice ($P<0.01$ low versus high; Figure 3C). In the KO mice, the basal [Ca$^{2+}$] was similar to the WT mice when the tubules were
perfused with low viscosity, but the shear stress–induced increase in [Ca2+]i was blocked both in the macula densa and TAL.

**MMDD1 Cell**

We previous found that tubular flow–induced NO generation by the macula densa was mediated by shear stress. To further determine whether shear stress increases intracellular calcium concentration ([Ca2+]i), we measured [Ca2+]i with fura-2 in cultured MMDD1 cells, a macula densa–like cell line. When we increased shear stress from 0.5 to 5 dynes/cm2, [Ca2+]i raised from 123±27.4 to 359±52.6 nmol/L (P<0.01; Figure 3A).

To determine the role of primary cilia in shear stress–induced [Ca2+]i, we applied siRNA against IFT88 to remove cilia on the MMDD1 cells. Removal of cilia inhibited the shear stress–induced calcium increase, which was from 115±21.3 to 167±41.9 nmol/L when we increased shear stress from 0.5 to 5 dynes/cm2 in the siRNA-treated cells (Figure 3B).

To determine the source of shear stress–induced calcium increase, we inhibited intracellular inositol triphosphate (IP3) calcium stores or chelated extracellular calcium. Phospholipase C inhibitor U-73122 and IP3 receptor inhibitor 2-APB (2-aminoethyl diphenylborinate) had no effect on shear stress–induced increase in [Ca2+]i. When we used calcium-free solution plus 5 µmol/L EGTA (ethylene glycol-bis-β-aminoethyl ether-N,N,N',N'-tetraacetic acid), the shear stress–induced [Ca2+]i elevation was inhibited (Figure 3C).

**Measurement of TGF Responsiveness In Vitro and In Vivo**

The TGF response in vitro was measured in the double-perfused JGA in response to an increase in the NaCl concentration of the tubular perfusate from 10 to 80 mmol/L in the KO and WT mice. The TGF was 2.8±0.2 µm (the afferent arteriole constricted by 16.1±1.1% from 17.4±1.2 to 14.6±0.9 µm) in the WT mice (Figure 4). The TGF response significantly enhanced to 4.2±0.3 µm in the KO mice (the afferent arteriole constricted by 25.5±1.7% from 16.5±1.3 to 12.3±0.8 µm; P<0.05 versus WT; Figure 4A and 4B).

TGF responses were also assessed in vivo by measuring the changes in proximal tubular stop flow pressure.
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(ΔPsf) after an increase of perfusion rate in the late proximal tubules. When the tubular perfusion rate was increased from 0 to 40 nL/min, Psf decreased by 16.7±1.5% from 35.4±2.6 to 29.5±1.7 mm Hg and the ΔPsf was 5.9±0.3 mm Hg in the WT mice. In the KO mice, Psf decreased by 22.9±1.8% from 36.7±3.1 to 28.3±2.4 mm Hg when the tubular perfusion rate was increased from 0 to 40 nL/min, and the ΔPsf was 8.4±0.5 mm Hg. TGF was significantly enhanced to 8.4±1.3 mm Hg in the KO mice versus 5.9±0.3 mm Hg in the WT animals (P<0.05; Figure 4C and 4D).

Measurement of GFR in Conscious Mice

GFR in conscious mice was measured using method of a single bolus injection of FITC–inulin as we described previously.20,30 The whole GFR and the GFR normalized by body weight showed no significant difference between the WT and KO mice. A high salt diet for 2 weeks did not significantly alter GFR in either the WT or the KO mice (Figure 5).

Comparison of the Natriuretic Response to Acute Volume Expansion in KO and WT Mice

To determine whether deletion of the primary cilia from the macula densa and TAL affects renal hemodynamics and sodium excretion, we measured the kidney clearance function after acute volume expansion by intravenous infusion of saline in WT and KO animals.

The MAP was normal and constant at baseline and during volume expansion in both WT and KO mice (Figure 5C).

The baseline GFR was similar in the WT and KO mice. The GFR rose by 1.2-folds (from 0.53±0.036 to 1.20±0.07 mL min⁻¹ g⁻¹ kidney weight; P<0.01 versus basal) in WT mice during 0 to 60 minutes after acute volume expansion. In contrast, GFR rose only by 54% in the KO mice (from 0.55±0.04 to 0.85±0.06 mL min⁻¹ g⁻¹ kidney weight; Figure 5D).

Baseline urinary flow rate and sodium excretion rate were similar in the WT and KO mice. The rate of urinary flow and sodium excretion increased by 3-folds in the WT mice in the first hour after acute volume expansion. In contrast, the

Figure 4. Measurement of tubuloglomerular feedback (TGF) response in vitro and in vivo. The TGF response in vitro was measured in isolated perfused juxtaglomerular apparatuses (JGAs), in which the tubules and the afferent arterioles were perfused simultaneously. The TGF response was determined by measuring diameter of the afferent arteriole when tubular perfusate was switched from 10 to 80 mmol/L NaCl solution and compared between wild-type (WT; n=7) and knock-out (KO; n=5) mice (A and B; *P<0.01 vs WT). The TGF response in vivo was measured with micropuncture. The TGF response was determined when the tubular perfusion rate was increased from 0 to 40 nL/min and compared between WT (n=13 tubules per 5 mice) and KO (n=11 tubules per 5 mice) mice (C and D; *P<0.05 vs WT).
diuretic and natriuretic responses were significantly blunted in the KO mice (Figure 5E and 5F).

Role of the Primary Cilia on the Macula Densa and TAL on Salt Sensitivity of Blood Pressure

To determine whether deletion of cilia from the macula densa and TAL promotes salt sensitivity of blood pressure, we compared changes in MAP measured by telemetry in WT and KO mice fed a high salt diet containing 4.0% NaCl. Baseline MAP measured in the mice fed a normal salt diet (0.4% NaCl) averaged 90.3±4.5 mm Hg in the WT mice and 91.4±4.1 mm Hg in the KO mice. After switching to a high salt diet, the MAP of the KO mice increased by 17.4±1.6 mm Hg, whereas it was not significantly altered in the WT mice (P<0.01 versus...
mary cilium is present on the apical surface of each epithelial cell and only about 20 to 30 macula densa cells in each JGA, therefore, the cilia will not always be visualized on the macula densa on a given kidney slice. Because of this, we conducted immunofluorescence on the isolated perfused JGA and clearly visualized the primary cilia on the macula densa. We confirmed the deletion of the primary cilia on the macula densa in the KO mice. To our surprise, we did not observe any renal cysts in the KO mice. The reasons might be because of either the cilia on the macula densa and TAL are not essential for the development of PKD, or not complete deletion of the cilia in the present model.

In this study, we measured NO generation by the macula densa and TAL, respectively. To avoid the potential interaction of the NO produced between the macula densa and TAL, we perfused from the distal tubule when we measured the macula densa NO generation, while perfusing the TAL when we measured the NO generation by the TAL. To determine the source of NO generation, we applied a selective NOS1 inhibitor 7-NI and a nonselective NOS inhibitor L-NAME. We previously reported that the tubular flow stimulates the primary cilia on the macula densa that enhances NOS1 activation and increases NO generation. In this study, we further confirmed that the macula densa NO generation reduced by 56% in the cilia of KO mice. Both 7-NI and L-NAME blocked the NO generation by the macula densa, indicating that the macula densa NO generation is the primary source of NO generation by the macula densa.12 These results are consistent with the previous reports from our laboratory and other investigators.12,20,29,30,38

The role of the cilia in NO generation in TALs has not been clarified. Luminal flow in the TAL has been found to stimulate NOS3 activity.12,39,40 However, it is not clear whether the flow-induced NOS3 activation in the TAL is mediated by the primary cilia. We concluded that the primary cilium play an essential role in the NO generation by the TAL. These findings provide a potential mechanism by which primary cilium on the TAL contribute to the tubular flow-induced NO generation.

We previously reported that shear stress enhanced NO generation by the macula densa.12 Because both NOS1 and NOS3 are calcium dependent constitutively expressed enzymes,12,39,42 we examined whether primary cilia had any effect on [Ca2+]i. To avoid the potential confounding effect of NaCl,29,43 we did not alter NaCl concentration in the perfusate with a constant tubular perfusion rate. We found that increase in shear stress significantly raised [Ca2+]i by 60% in the macula densa and by 33% in TAL. The shear stress–induced [Ca2+]i alterations were blocked in the KO mice, indicating that the primary cilium negate the shear stress–induced intracellular calcium changes.

We recently found that shear stress stimulates macula densa NOS1 activity mediated by the primary cilium.12 We previously reported that NOS1β is the primary splice variant and contributes to most of the NO generation by the macula densa, which inhibits the TGF response.16,17 Our laboratory recently demonstrated the physiological significance of TGF responsiveness in the long-term control of hemodynamics, in which the mice with deletion of NOS1 specifically from the macula densa developed salt-sensitive hypertension, associated with enhanced TGF response and impaired sodium excretion.20 Therefore, it is intriguing to know whether the macula densa NOS1-induced changes in TGF response and hemodynamics

**Discussion**

In this study, we demonstrated that the primary cilia on the macula densa and the TAL play an essential role in the development of salt-sensitive hypertension by regulation of the TGF response, the GFR, and the sodium excretion. In response to acute volume expansion, the elevation in GFR and sodium excretion were blunted in the KO mice. These KO mice exhibited enhanced TGF response both in vivo and in vitro. In response to acute volume expansion, the elevation in GFR and sodium excretion were blunted in the KO mice. The blood pressure in the KO mice significantly increased after a high salt diet. We confirmed the deletion of the primary cilia on the macula densa in the KO mice. To our surprise, we did not observe any renal cysts in the KO mice. The reasons might be because of either the cilia on the macula densa and TAL are not essential for the development of PKD, or not complete deletion of the cilia in the present model.

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in vivo are modulated by the primary cilia. In this study, we
generated a tissue-specific primary cilia deletion mouse line,
in which the primary cilia are deleted from the macula densa
and TALs. The TGF response was enhanced both in vivo
and in vitro in the KO mice. The baseline blood pressure and GFR
in conscious mice were at similar levels in both the WT and
the KO mice, which may reflect the net results of compensa-
tory effects. Therefore, we tested the responses after acute salt
loading. In response to acute volume expansion, the KO mice
exhibited lower GFR elevation and impaired sodium excretion
compared with the WT mice. After a high salt diet, the MAP
in the KO mice was significantly higher. These data indicate
that deletion of the primary cilia from the macula densa and
the TAL induces salt-sensitive hypertension associated with
inhibited GFR elevation and impaired sodium excretion.

However, we are unable to differentiate the role of the
primary cilia on the macula densa from that on the TAL because
no specific marker for the macula densa is available. We dem-
onstrated in this study that deletion of the primary cilia from
the TAL decreased NO generation by the macula densa.
It has been well established that the NO produced by TAL
inhibits sodium reabsorption by inhibition of NKCC2 and sodium-hydrogen
echanger cotransporter activities in the TAL, which promotes
sodium excretion. Meanwhile, this effect should increase
sodium concentration at the macula densa, consequently, it
should enhance TGF response. However, another elegant study
using isolated perfused JGAs reported that the NO produced
by TAL acts as a paracrine factor and signals the macula densa
that inhibited the TGF response. Thus, the net effect of
the NO produced by the TAL on the TGF response is complicated
and not conclusive. In addition, whether primary cilia on the
TAL have any direct effect on the activity of the cotransporters
remains to be determined.

In summary, we developed a tissue-specific primary cilia
deletion mouse strain, in which the primary cilia were removed
from the macula densa and the TAL. These KO mice exhib-
ited reduced NO generation by the macula densa and TAL and
enhanced TGF response in vivo and in vitro. In response to an
acute volume expansion, the elevation in GFR was limited,
and sodium excretion was impaired in the KO mice compared
with the WT mice. The KO mice developed salt-sensitive
hypertension. On the basis of our data, we conclude that pri-
mary cilia on the macula densa and TAL play an important
role in the pathogenesis of salt-sensitive hypertension.

Perspectives
A possible mechanism is proposed based on the results of
this study that the tubular flow stimulates the primary cilia
via shear stress. This enhances NOS1 activity in the macula
densa by elevation of [Ca\(^{2+}\)], which blunts TGF responsive-
ness leading to an increase in GFR, promoting sodium excre-
tion and contributing to electrolyte and volume homeostasis.
Impairment of this signaling pathway would promote sodium
retention and contribute to the development of salt-sensitive
hypertension.

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Disclosures
No conflict of interest.

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**Novelty and Significance**

**What Is New?**

- A new animal model with tissue-specific cilia deletion mouse strain was developed; a new mechanism for regulation of hemodynamics mediated by the primary cilia on the macula densa and thick ascending limb was determined.

**What Is Relevance?**

- This study provides a novel mechanism for the significance of the primary cilia on the macula densa and thick ascending limb in the development of salt-sensitive hypertension.

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**Summary**

The primary cilia on the macula densa and thick ascending limb play an important role in the control of sodium excretion and maintain normal hemodynamics.
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Role of the primary cilia on the macula densa and thick ascending limbs in regulation of sodium excretion and hemodynamics

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Running title: Primary cilia on the macula densa and TAL and hypertension

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SUPPLEMENTAL DATA:

METHODS

Microperfusion

The Af-Art and attached macula densa were isolated and microperfused as previously described. Briefly, mice were anesthetized with inhaled isoflurane (Butler chemicals, UK). The kidneys were removed and sliced along the corticomedullary axis. Slices were placed in ice-cold minimum essential medium (MEM; Gibco, Grand Island, NY) containing 5% bovine serum albumin and dissected under a stereomicroscope (model SMZ 1500; Nikon). From each mouse, a single superficial Af-Art and its intact glomerulus were microdissected together with adherent tubular segments consisting of portions of the thick ascending limb of the loop of Henle, the macula densa, and the early distal tubule. Using a micropipette, the microdissected complex was transferred to a temperature-regulated chamber mounted on an inverted microscope (Eclipse Ti; Nikon) with Hoffmann modulation. Both the Af-Art and the end of either the distal tubule or thick ascending limb were cannulated with an array of glass pipettes. The Af-Art was perfused with MEM, and the intraluminal pressure was maintained at 60 mm Hg throughout the experiment. The macula densa was perfused with physiologic saline consisting of (in mM) 10 HEPES; 1.0 CaCO3; 0.5 K2HPO4; 4.0 KHCO3; 1.2 MgSO4; 5.5 glucose; 0.5 Na acetate; 0.5 Na lactate, L-arginine 0.5; and either 80 NaCl or 10 NaCl. The pH of the solution was 7.4. The perfusion bath was exchanged continuously at a rate of 1 mL/min. Microdissections were completed within 30 minutes at 3-5°C, and the samples were then transferred into the bath and gradually warmed to 37°C for the rest of the experiment. A 30-minute equilibration period was allowed before taking any measurements. The imaging system consisted of a microscope (Eclipse Ti; Nikon), a digital charge-coupled device camera (CoolSnap; Photometrics), a xenon light (LB-LS/30; Shutter Instruments), and an optical filter changer (Lambda 10–3; Shutter Instruments). Images were displayed and analyzed with NIS-Elements imaging software (Nikon).

Identification of primary cilia on macula densa cells in the isolated perfused JGA

We detected primary cilia on the macula densa with immunofluorescence in the isolated perfused rabbit JGA as we previously reported. Briefly, the JGA was isolated, and the TAL was cannulated and perfused for 30 min. The macula densa was then fixed by adding 4% paraformaldehyde in phosphate-buffered saline (PBS) to both bath and lumen for 30 min and washed for 10 min by adding Tris-buffered saline Tween-20 (TBST). The sample was permeabilized and blocked with 0.1% Triton X-100 and 3% BSA for 60 min. Cilia were immunolocalized by perfusing a primary antibody against acetylated tubulin for 60 min (Santa Cruz 6-11B-1; 1:800), and then an Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody for 30 min (Invitrogen; 1:400). Cilia on the macula densa were visualized with fluorescence microscopy.

Immunofluorescence

Similar methods were used as we previously reported. Kidneys were collected and perfused with saline, followed with 10% buffered formalin solution. The tissues were further fixed overnight and then embedded in paraffin. After deparaffinization and antigen
unmasking, the sections were blocked with 10% normal goat serum and 0.1% Tween-20 in PBS for 1 h. For the detection of NKCC2, the slices were then incubated overnight with an NKCC2 antibody (Rabbit polyclonal IgG, 1:1000, obtained from Dr. Pablo Ortiz, Henry Ford Hospital, Detroit, MI). After washing, the sections were incubated for 1 hour with a fluorescent secondary antibody (Light 488-conjugated AffiniPure Goat anti-Rabbit, 1:2000, Jackson ImmunoResearch Laboratories, West Grove, PA). For the detection of the primary cilia, the slices were incubated with a primary antibody against acetylated α-tubulin (Santa Cruz 6-11B-1, 1:2000, Santa Cruz Biotechnology, Santa Cruz, CA) overnight followed with a fluorescent secondary antibody (Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody, 1:1000; Invitrogen, Waltham, MA) for 1 hour. Negative controls included sections incubated without primary and/or secondary antibodies. All slices were mounted with anti-fade medium (Jackson Immuno Research Laboratories, West Grove, PA) and images were captured on an imaging system (Nikon Eclipse Ti, Nikon Instruments Inc., Melville, NY).

**Measurement of NO in isolated perfused JGA**

We measured NO production in the macula densa and the TAL using a cell permeable fluorescent NO indicator 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA ) as previously described. Briefly, the tubules were loaded with 10 µM DAF-FM DA in 0.5% dimethyl sulfoxide plus 0.1% pluronic acid from the tubular lumen for 30 to 40 min, then washed for 10 min. Tubules was perfused with 80 mM NaCl solution at a rate of 10 nL/min with a pump. DAF was excited at 490 nm with a xenon light, and the emitted fluorescence was recorded at wavelengths of 510 to 550 nm. Square-shaped regions of interest (ROIs) were set inside the cytoplasm of macula densa cells and TALs. The mean intensity within the ROI’s was recorded every 5 sec for 5 min. NO production was calculated based on the percentage changes in the basal fluorescence intensity of DAF.

**Isolation of macula densa cells**

Laser capture microdissection (LCM) was used to isolate macula densa cells from frozen kidney slices, as we previously described. Kidneys from mice were removed and snap-frozen in Tissue-Tek Optimal Cutting Temperature Compound (Thermo Fisher Scientific, Waltham, MA). Frozen sections (8 µm) were prepared and then stained and dehydrated using an Arcturus Histogene frozen section staining kit (Life Technologies, Carlsbad, CA). The macula densa cells were dissected using a Laser Capture Microdissection System (Arcturus, Grand Island, NY).

**Real-Time PCR**

RNA was extracted from the LCM isolated macula densa cells using a PicoPure RNA isolation kit (Life Technologies, Carlsbad, CA). Quantitative PCR analysis was performed using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) and CFX96 Real-Time Detection System (Bio-Rad, Hercules, CA). The Real-Time PCR reactions contained 1µl of the RT reaction and 0.1µM of the forward and reverse primers in 25µl volume. The cycling conditions were: 1 cycle at 95ºC for 3min, followed by 40 cycles at 95ºC for 30s, 60ºC for 30s, and 72º C for 30s. The relative expression of the NOS1 splice
variants was compared using the delta CT method. The following primers were used to amplify the various splice variants: NOS1α: 5'-GCTCGGCAGCAGCTCCAGGTA-3', and 5'-TCAAGGTTGACCAGGCAGACG-3'; NOS1β: 5'-GGGCTCGGCAGCAGAGACCTC-3', and 5'-GTCAGGAAGGTCCACGGG-3'. The GAPDH was used as a housekeeping gene and the primers sequences were: 5'-TGATGACATCAAGAAGGGGGAA-3', and 5'-TCCTTGAGGCATGTGGGCC-3'.

**Western blot to measure splice variants of NOS1**

Renal cortical protein extracts (50μg/lane) were separated on a 7.5% SDS-PAGE gel. After blocking for 1 hour at room temperature with 5% skim milk, the membranes were incubated overnight at 4 °C with a C-terminal NOS1 antibody (Mouse polyclonal IgG, 1:3000, Bd Biosciences, San Jose, CA). After incubation of the membranes with horseradish peroxidase conjugated secondary antibody (goat anti-mouse, IgG, 1:300000, Bio-Rad, Hercules, CA), the immunoreactive bands were revealed by enhanced chemiluminescence detection on Hyperfilm (Amersham Pharmacia Biotech, Piscataway, NJ) and normalized by β-actin.

**Measurement of [Ca^{2+}]_i in isolated perfused JGAs and cultured MMDD1 cells**

**Intracellular calcium measurement:** Similar methods were used as we previously described. The perfused JGA or the cultured cells were loaded with 3 μM fura-2 AM for 30 min in Ringer’s solution (3 μM in 0.1% DMSO) and then washed for 20 min. [Ca^{2+}]_i will be calculated from the equation:

\[
[Ca^{2+}]_i = K_d \times \frac{(R - R_{min})}{(R_{max} - R)} \times \left(\frac{F_o}{F_s}\right)
\]

where \(K_d\) is the dissociation constant of fura-2, \(R\) is the ratio of fluorescence at 380 to 340 nm, and \(R_{min}\) and \(R_{max}\) are the ratios for unbound and bound forms of the fura-2/Ca^{2+} complex in the cells, respectively. \(F_o/F_s\) is the ratio of fluorescence at 380 nm in the absence of Ca^{2+} to saturating Ca^{2+} concentrations. We have calibrated the system in situ.

**Shear stress adjustment in isolated perfused JGAs:**

The viscosity of the tubular perfusate was increased by adding a high molecular weight dextran (MW: 200,000, MP Biomedicals, Solon, OH) to a perfusate containing 80 mM NaCl solution as we reported previously. Viscosity was measured at 37° C with a glass capillary viscometer (Cannon Instrument Company, State College, PA). Osmolalities of the solutions without dextran were adjusted with mannitol to achieve the same osmolality as the solution with dextran. We shortened distal tubule to <10 μm to minimize the changes in perfusion pressure induced by increase in viscosity.

[Ca^{2+}]_i in the macula densa and TAL was measured for 5 min at a low shear stress. Then shear stress was increased to a high shear stress for 10 min, and [Ca^{2+}]_i was measured again for 5 min. Timed control experiments were performed using the same protocol without changing the shear stress.

**MMDD1 cells:** Experiments were also performed using MMDD1 cells, a macula densa-like cell line (kindly provided by Dr. J. Schnermann, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD). Similar methods were used for MMDD1 cell culture, shear stress adjustment, siRNA treatment and [Ca^{2+}]_i measurement.
as we previously described\textsuperscript{3,11,12,14}. MMDD1 cells at passages 20–25 were cultured on sterilized coverslips in DMEM nutrient mixture-Ham’s F-12 (DMEM/F12, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin) and incubated in a humidified atmosphere of 95% room air and 5% CO\textsubscript{2} at 37°C.

**Preparation of small interfering RNA (siRNA):** Primary cilia function was disrupted by knocking down the expression of the ift88 gene with a siRNA. MMDD1 cells were transferred onto 6-well plates (about 5 x 10\textsuperscript{5} cells per well) and incubated for 24 h before transfection. A siRNA targeting ift88 (sequence: 5’-AAAG GUCG UCCU CAUC UGUU UCUG G-3’) or a scrambled control siRNA (Invitrogen) was transfected into cells using XtremeGene (Roche Molecular Systems, Alameda, CA) according to the manufacturer’s instructions. The final concentration of siRNAs in the bath was 30 nM. The medium was changed 24 h after transfection and the flow experiments were performed at 48 h. The effectiveness of the siRNA was confirmed by measuring the expression of ift88 mRNA using real-time PCR and by immunofluorescence imaging of primary cilia.

**Shear stress adjustment in MMDD1 cells:** A circular flow chamber system (GlycoTech, MD) was used to quantitatively increase shear stress in MMDD1 cells. The coverslips were sealed in the flow chamber which was connected with a pump. Shear stress was adjusted by changing the flow rate in the chamber.

After a 20 min equilibration period, [Ca\textsuperscript{2+}]\textsubscript{i} was measured for 5 min at shear stress of 0.5 dynes/cm\textsuperscript{2}. Then shear stress was increased to 5.0 dynes/cm\textsuperscript{2} for 10 min, and [Ca\textsuperscript{2+}]\textsubscript{i} was measured again for 5 min. Timed control experiments were performed using the same protocol without changing the shear stress.

**GFR measurement in conscious mice**

We used a single bolus injection of FITC-inulin, similar to a previously published method\textsuperscript{6,18} for measurement of GFR in conscious mice. Dialyzed FITC-inulin (5% in saline, 3.74µL/g body weight) was injected via the penile vein. The mice were lightly anesthetized with isoflurane during injection and awakened within a minute. Blood (20 µL) was collected into heparinized capillary tubes at the following times: 3, 7, 10, 15, 35, 55, 75, and 90 minutes via the tail vein. The fluorescence of FITC-inulin in the plasma was detected by the plate reader, and the GFR was calculated with a GraphPad Prism.

**Renal clearance in response to isotonic volume expansion**

Methods utilized for measurement of kidney clearance function were similar to those that we recently described\textsuperscript{6,18}. At the end of 7-NI treatment or high salt diet, the mice were anesthetized with ketamine (30 µg/g) and inactin (50 µg/g), and a catheter was placed in the femoral vein for an intravenous infusion of 2% BSA and FITC-inulin (2mg/ml) in a 0.9% NaCl solution at a rate of 0.5 ml/hour. Another catheter was inserted into the left ureter for urine collection. After surgery, urine and plasma were collected during a 30-min period after a 30-min equilibration period. This was followed by a bolus infusion of saline (3% body weight), and the infusion rate was maintained at 0.5 ml/hour with FITC-inulin afterward. Urine and plasma were collected during a 60-min period and a 61-90 min period after volume expansion. At the end of the experiment, the left kidney was removed and weighed, and the concentrations of Na\textsuperscript{+} and inulin in the urine and plasma samples were determined.

**Micropuncture**
Methods for animal preparation were the same as we previously published\textsuperscript{2,19,20}. The Mice were anesthetized with inactin (I.P. 70mg/Kg) and ketamine (I.M., 50mg/Kg). Body temperature was maintained at approximately 36.5°C by a heated operating table controlled by feedback from a rectal thermometer (Vestavia Scientific, Birmingham AL). A tracheal cannula was inserted with PE90 tubing for unrestricted respiration. The right carotid artery was catheterized for continuous recording of blood pressure by a pressure transducer. The jugular vein was catheterized for sustaining infusion of a 0.9%NaCl saline with 2.25% bovine serum albumin at 0.4 ml/hr 30g BW\textsuperscript{-1}. The bladder was catheterized via a suprapubic incision along the linea alba. The left kidney was exposed by a flank incision, dissected free from its peri-renal attachments, laid in a kidney cup, and embedded with 4% agar in saline. The renal surface was bathed in saline. After an equilibration period of 30-45 min, a random proximal tubular segment was punctured with a micropipette (o.d., 6-8 µm) filled with artificial tubular fluid (ATF) (containing in mM: 4 NaHCO\textsubscript{3}, 5 KCl, 2 CaCl\textsubscript{2}, 7 Urea, 2 MgCl\textsubscript{2}, 128 NaCl, pH 7.4) and stained with 0.1% fast green. Superficial segments of the proximal convoluted tubule were identified by injection of fast green solution. A grease pipette (o.d., 10µm) was then inserted into the same site, and flow was blocked by injection of a grease block. A perfusion pipette (o.d., 6-8µm) containing artificial tubular fluid plus dye was inserted into the last segment of the late proximal tubule downstream from the grease block and connected to a nanoliter microperfusion pump (Vestavia Scientific, Birmingham, AL). A pressure pipette (o.d., 1-2µm), filled with 2mol/L KCl and connected to a micropressure system (model 900A, World Precision Instruments, Sarasota, FL), was inserted upstream from the grease block to measure proximal stop-flow pressure (P\textsubscript{sf}). When the perfusion rate was switched from 0 nL/min to 40 nL/min, a delta change of P\textsubscript{sf} was recorded as an indicator of TGF. The P\textsubscript{sf} and the mean arterial pressure (MAP) were recorded and displayed by a PowerLab data acquisition system (ADInstruments, Colorado Springs, CO). At the end of each experiment, an arterial blood sample was taken with a heparinized capillary tube to measure hematocrit.

Telemetry transmitter implantation

Similar methods for transmitter implantation and MAP monitoring were used as we described previously\textsuperscript{6,18,21}. Mice were anesthetized with inhaled isoflurane (Butler chemicals, UK). A small incision was made in the middle of the neck for insertion of the telemetry transmitter (PA-C10, Data Sciences International). The catheter of the transmitter was placed in the left carotid artery and advanced to the aortic arch. The body of the transmitter was placed subcutaneously in the right ventral flank of the mice. The mice were allowed to recover for 10 days. The MAP was measured for 30 seconds every 10 min continuously.
Reference List:


Fig S-1a-Systolic blood pressure

Fig S-1b-Diastolic blood pressure
Fig S-1c – Heart rate

![Heart rate graph showing mean arterial pressure over time for WT and KO mice under normal and high salt conditions.](image-url)

- Normal Salt
- High Salt
- WT
- KO