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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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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 knockout (KO) mouse line was generated by crossing NKCC2-Cre mice with IFT88-Δ/flox mice (NKCC2^{CRE}; IFT88Δ$^{/flox}$), 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.[1,5,13] 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.[12] 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

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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 C57BL/6 background were used as control for the knock-out (KO) mice.

Microperfusion

The afferent arteriole and attached macula densa were isolated and microperfused as described previously[12,17,20] and described in online-only Data Supplement.

Immunofluorescence

Similar methods were used as we previously reported[12,20,21] 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 cilia on the macula densa with immunofluorescence in the isolated perfused rabbit juxtaglomerular apparatus (JGA) as we previously reported[12] 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′-difluorofluorescein diacetate as previously reported[12,20,22,23] and described in the 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[20,24,25] and described in the 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 $[Ca^{2+}]_i$ in Isolated Perfused JGAs and Cultured MMDD1 Cells

Intracellular Calcium Measurement

Similar methods were used as we previously reported[26–29] 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: 200 000; MP Biomedicals, Solon, OH) to a perfusate containing 80 mmol/L NaCl solution as we reported previously[12] 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 $[Ca^{2+}]_i$ measurement as we previously reported[12,26,27,29] 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[20,30] 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[20,30] and described in the online-only Data Supplement.

Micropuncture

Methods for animal preparation were the same as we previously published[20,31,32] 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[20,30,33] 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.[20] By crossing the NKCC2-Cre mice with IFT88-Δ/flox mice,[34,35] we generated a tissue-specific cilia deletion mouse line ($NKCC2^{CRE}$; $IFT88^{\Delta/flox}$, 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 NKCC2 as a marker for the macula densa and the TAL or 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 cilia were present in the lumen of the TAL (Figure 1Ac), 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 cilia 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

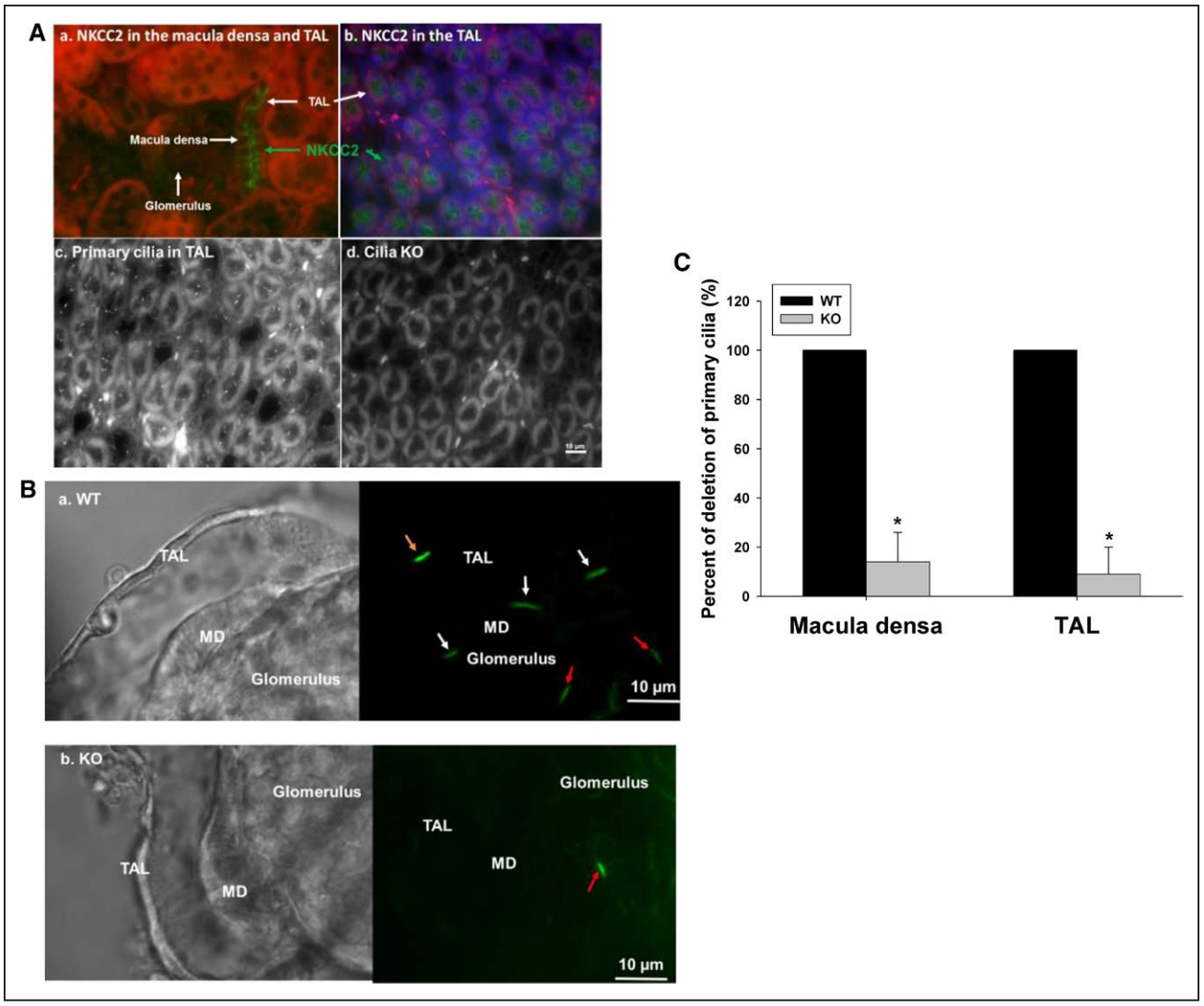

Figure 1. A, Immunofluorescent measurement of NKCC2 and primary cilia in the macula densa (MD) and thick ascending limb (TAL) in wild-type (WT) and knock-out (KO) mice. NKCC2 was detected using an NKCC2 antibody that marked the apical membrane of the macula densa and TAL in the renal cortex (a) and medulla (b). Primary cilia were detected with an antibody against acetylated α-tubulin and visualized in the lumen of the TAL in the renal medulla (c), which were absent in the KO mice (d). **B**, Immunofluorescent measurement of the primary cilia on the macula densa in isolated perfused juxtaglomerular apparatuses (JGAs) in WT and KO mice. Primary cilia were observed on the macula densa (white arrows) and TAL (orange arrow) isolated from WT mice (a). No primary cilia were detected on the macula densa and TAL isolated from KO mice. The red arrows indicates cilia in the glomerulus (b). The left side are brightfield images of the same perfused JGAs indicating the anatomic structures. **C**, Percent of deletion of the primary cilia in the KO mice. The number of primary cilia on the macula densa was determined in the isolated perfused JGAs with immunofluorescent method (n=8 WT, 7 KO mice). The primary cilia on TAL were determined with 3 kidney slices per mouse (n=5 WT, 5 KO mice). *P<0.01 vs WT.

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

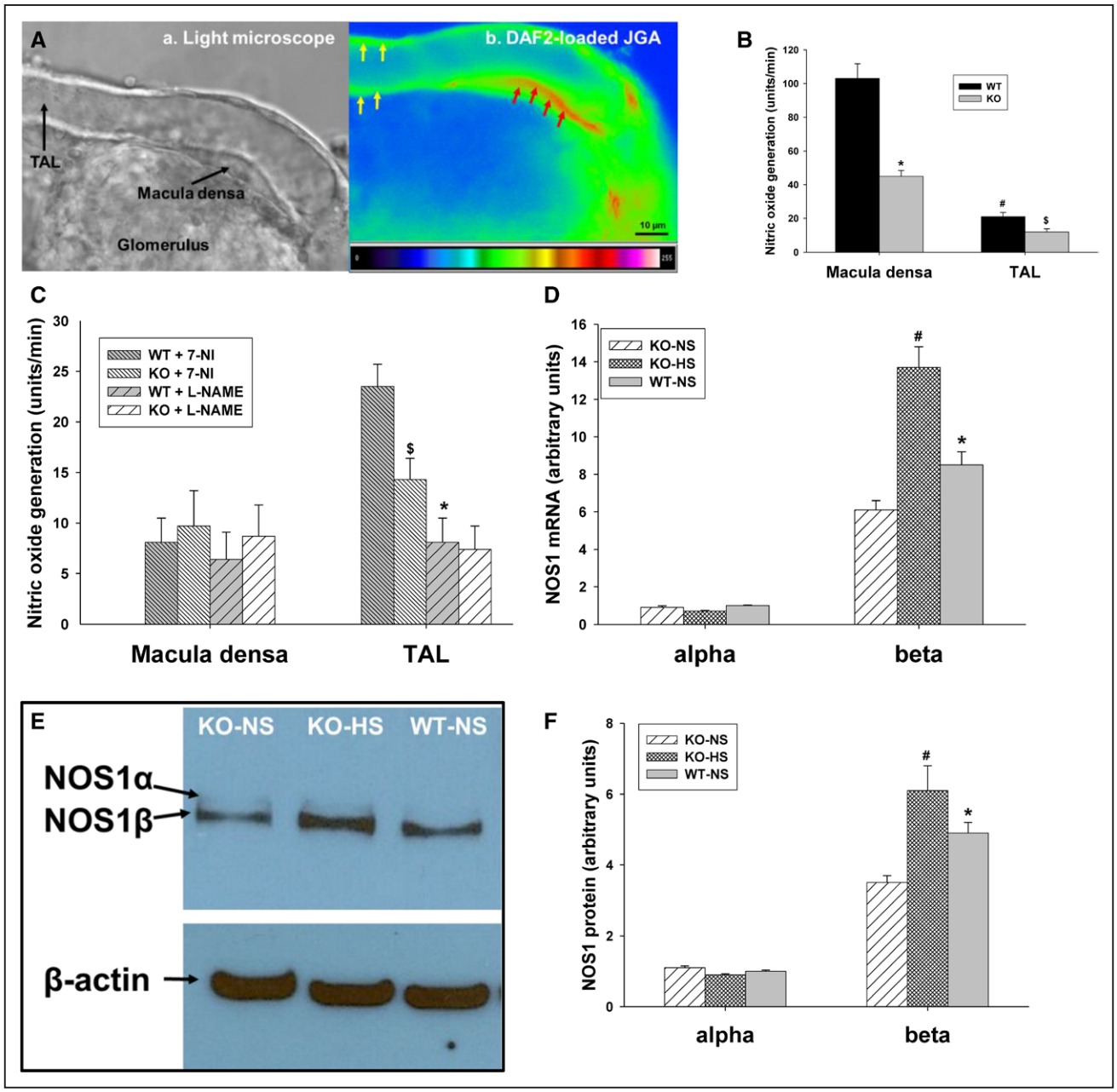

Figure 2. Measurement of NO generation by the macula densa and thick ascending limb (TAL) and nitric oxide synthesis (NOS1) splice variant expression levels in wild-type (WT) and knock-out (KO) mice. NO generation was measured in isolated perfused juxtaglomerular apparatus (JGA). **A**, (a) Brightfield image demonstrating the anatomic structure of the perfused JGA. **A**, (b) DAF-2 (4,5-diaminofluorescein diacetate) loaded JGA shown in a. NO generation by the macula densa (red arrows) and TAL (yellow arrows) was measured. **B**, Average data of NO generation by the macula densa and TAL in the WT (n=6) and KO (n=5) mice (**P*<0.01 vs WT; #*P*<0.01 vs macula densa NO [$*P*<0.05 vs WT]). **C**, Effect of selective NOS1 inhibitor 7-NI (n=7 WT, n=6 KO) or nonselective NOS inhibitor L-N^G-nitroarginine methyl ester (L-NAME; n=5 WT, n=5 KO) in NO generation by the macula densa and TAL in WT and KO mice (**P*<0.05 vs L-NAME). **D**, mRNA levels of NOS1 splice variants in the macula densa isolated with laser capture microdissection. **E**, **F**, Protein levels measured with a C-terminal NOS1 antibody. The KO mice were fed a high salt (HS) diet for 7 d (n=6) and compared with the KO (n=5) and WT (n=7) mice fed a normal salt (NS) diet (**P*<0.05 vs KO-NS and KO-HS, #*P*<0.01 vs KO-NS).

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 ($[Ca^{2+}]_i$), 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, $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ was similar to the WT mice when the tubules were

perfused with low viscosity, but the shear stress–induced increase in $[Ca^{2+}]_i$ 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.[12] To further determine whether shear stress increases intracellular calcium concentration ($[Ca^{2+}]_i$), we measured $[Ca^{2+}]_i$ with fura-2 in cultured MMDD1 cells, a macula densa–like cell line.[36,37] When we increased shear stress from 0.5 to 5 dynes/cm^2, $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$, 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/cm^2 in the siRNA-treated cells (Figure 3B).

To determine the source of shear stress–induced calcium increase, we inhibited intracellular inositol trisphosphate (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 $[Ca^{2+}]_i$. 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 $[Ca^{2+}]_i$ 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

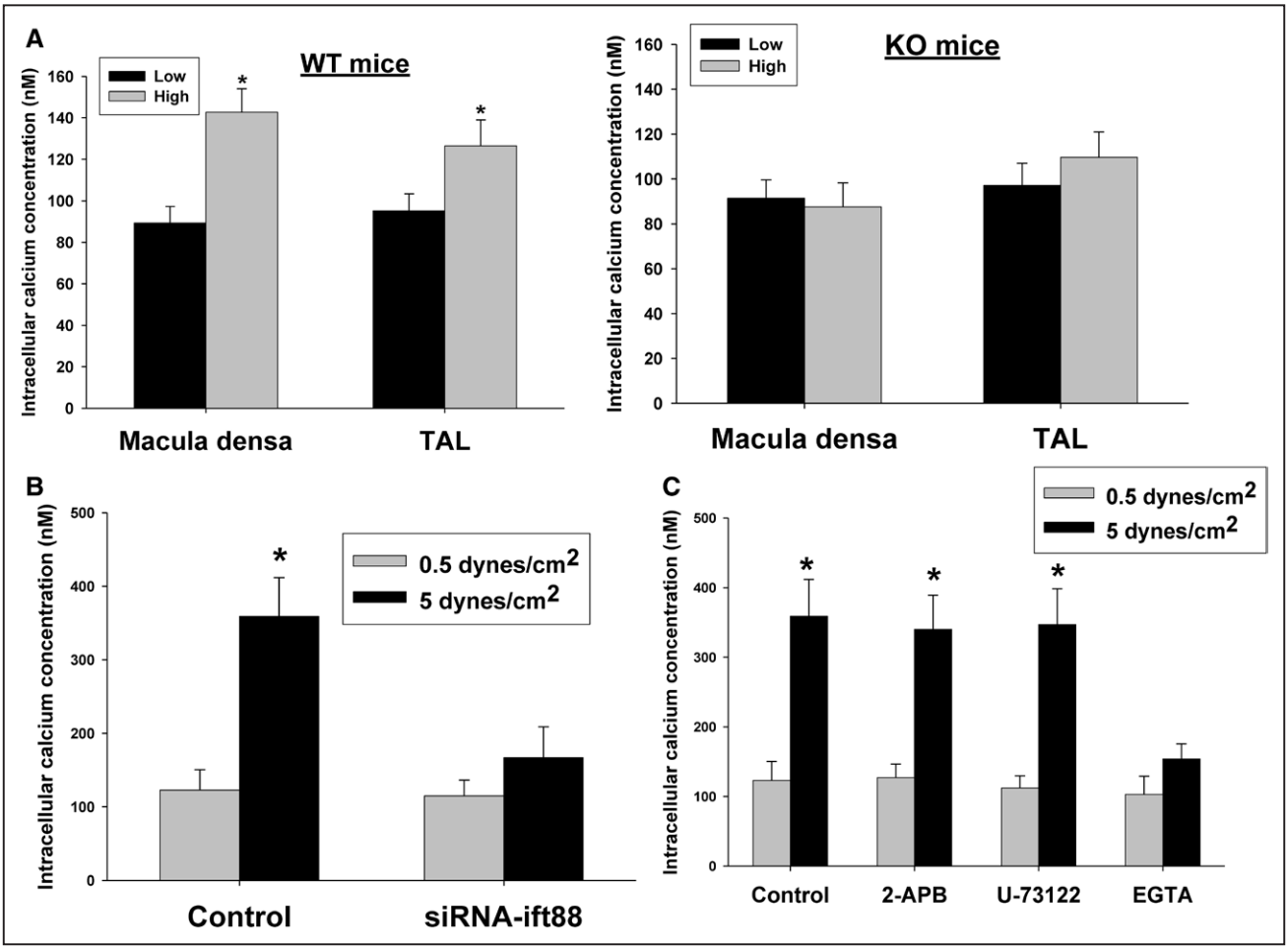

Figure 3. Measurement of intracellular calcium concentration in perfused juxtaglomerular apparatuses (JGAs) and in MMDD1 cells. Intracellular calcium concentration in isolated perfused JGAs and in cultured MMDD1 cells was measured with fura-2 AM in response to an increase in shear stress. **A**, Effect of shear stress adjusted by adding dextran in perfusate on intracellular calcium in the macula densa and thick ascending limb (TAL) in wild-type (WT) and knock-out (KO) mice (*<0.05 low vs high shear stress; n=5 WT, n=7 KO). **B**, Effect of shear stress increased from 0.5 to 5 dynes/cm^2 in control cells and in cells treated a siRNA to remove the primary cilia (*P<0.01 vs 0.5 dynes/cm^2, n=17 experiments for control, 12 experiments for siRNA). **C**, Effects of inositol trisphosphate inhibitor 2-APB (2-aminoethyl diphenylborinate), phospholipase C inhibitor U-73122, and extracellular calcium-free solution plus EGTA (ethylene glycol-bis-β-aminoethyl ether-*N,N,N′,N′*-tetraacetic acid) on the shear stress–induced calcium alterations (*<0.01 vs 0.5 dynes/cm^2, n=8 experiments for 2-APB, 14 for U73122 and 9 for EGTA).

(ΔP_{sf}) after an increase of perfusion rate in the late proximal tubules. When the tubular perfusion rate was increased from 0 to 40 nL/min, P_{sf} decreased by 16.7±1.5% from 35.4±2.6 to 29.5±1.7 mm Hg and the ΔP_{sf} was 5.9±0.3 mm Hg in the WT mice. In the KO mice, P_{sf} 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 ΔP_{sf} 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

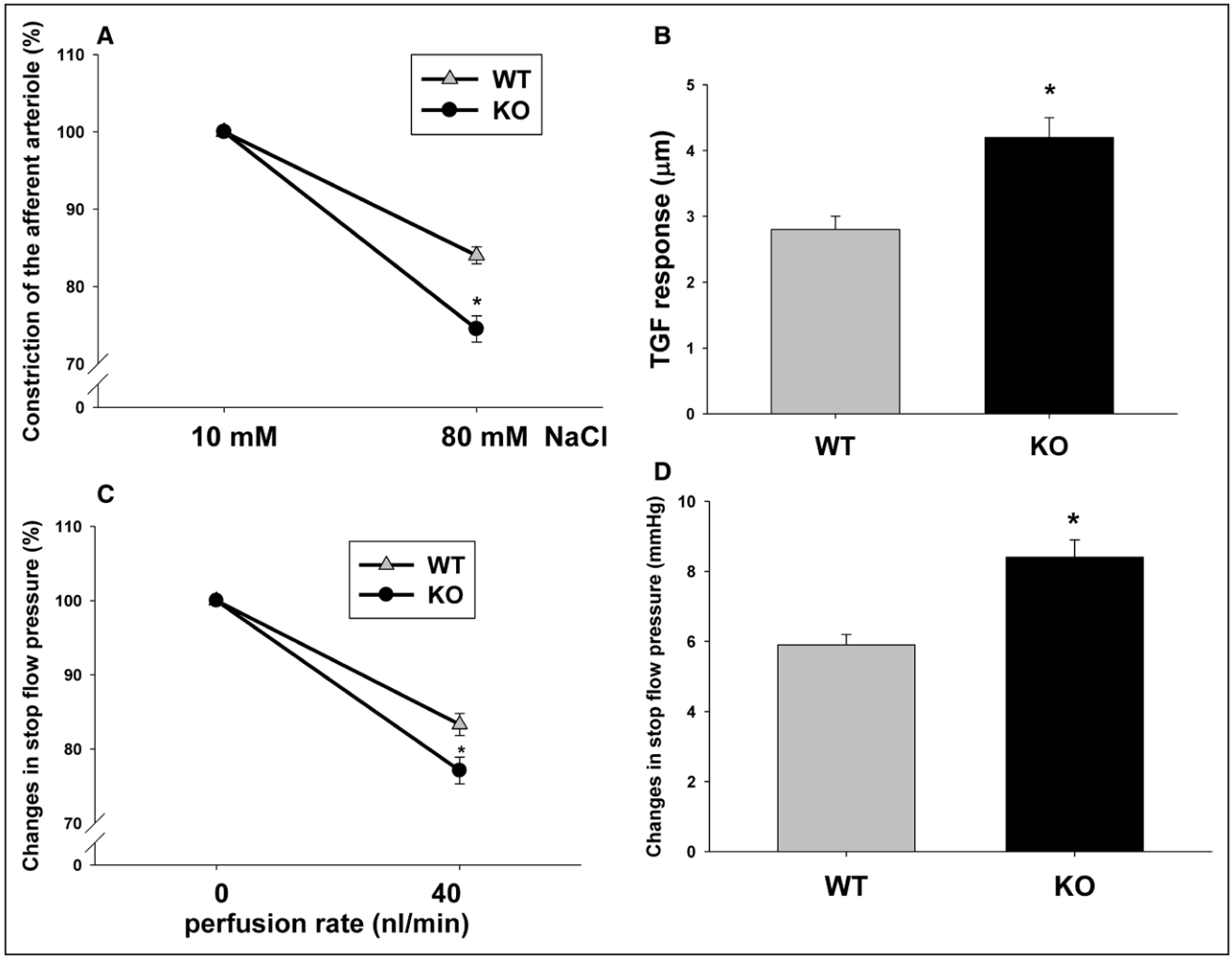

Figure 4. Measurement of tubuloglomerular feedback (TGF) response in vitro and in vivo. The TGF response in vitro was measured in isolated perfused juxtaglomerular apparatuses (JGAs), in which the tubules and the afferent arterioles were perfused simultaneously. The TGF response was determined by measuring 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).

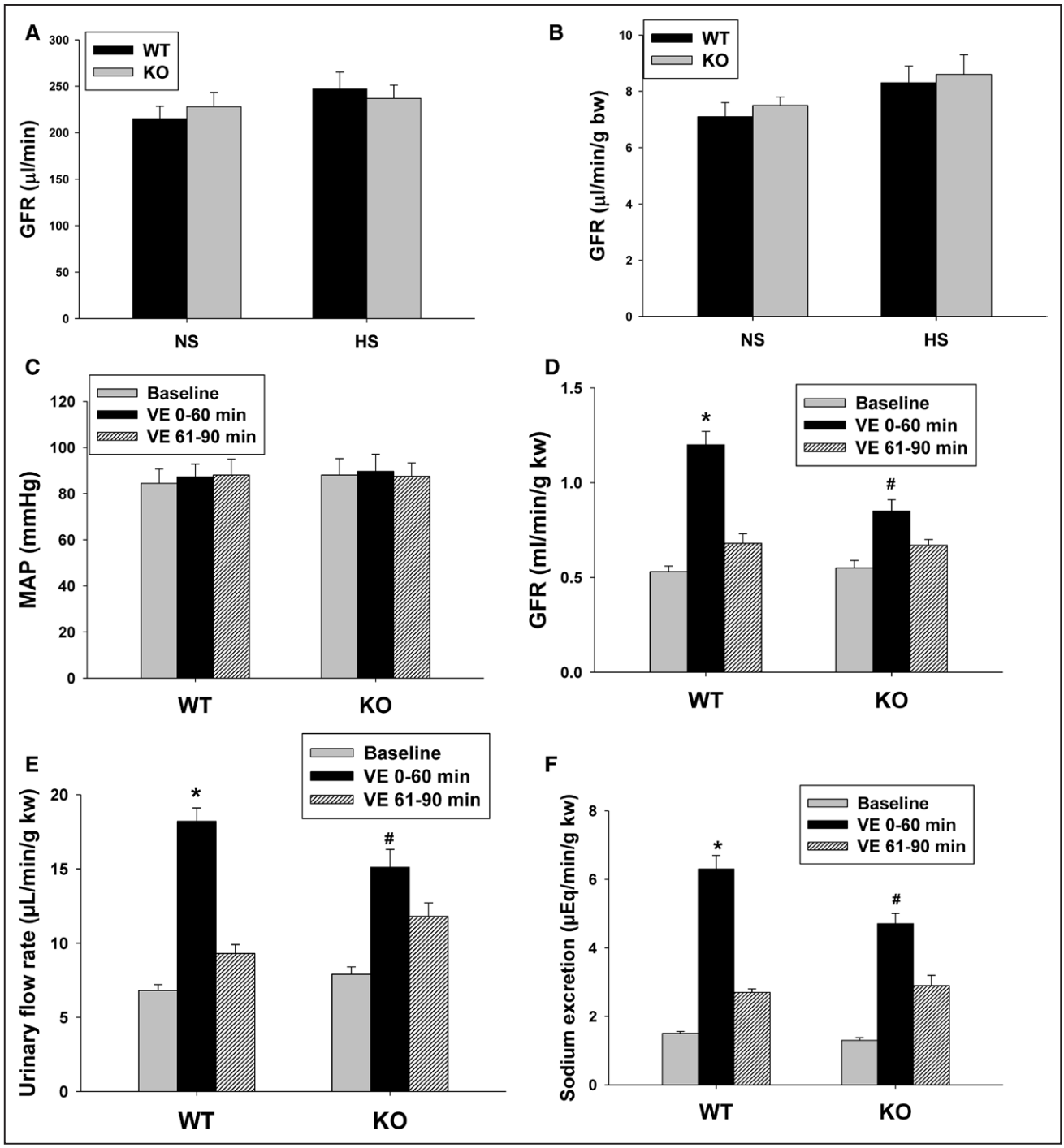

Figure 5. Measurement of glomerular filtration rate (GFR) in conscious mice and kidney clearance function in response to acute volume expansion. The GFR was measured in conscious mice using a single bolus injection of fluorescein isothiocyanate (FITC)–inulin. Whole GFR (**A**) and GFR normalized by body weight (BW; **B**) were determined between the wild-type (WT) and knock-out (KO) mice fed a normal salt diet (NS; n=8 WT; n=4 KO) or a 2-wk high salt diet (HS; n=5 WT; n=4 KO). Kidney clearance function was measured at baseline and at 30 to 60 min and 61 to 90 min after acute volume expansion in WT (n=6) and KO (n=7) mice. The mean arterial pressure (MAP; **C**), GFR (**D**), urinary flow rate (**E**), and sodium excretion rate (**F**) normalized by the kidney weight (KW) were measured. **P*<0.01 vs baseline, #*P*<0.01 vs baseline, *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

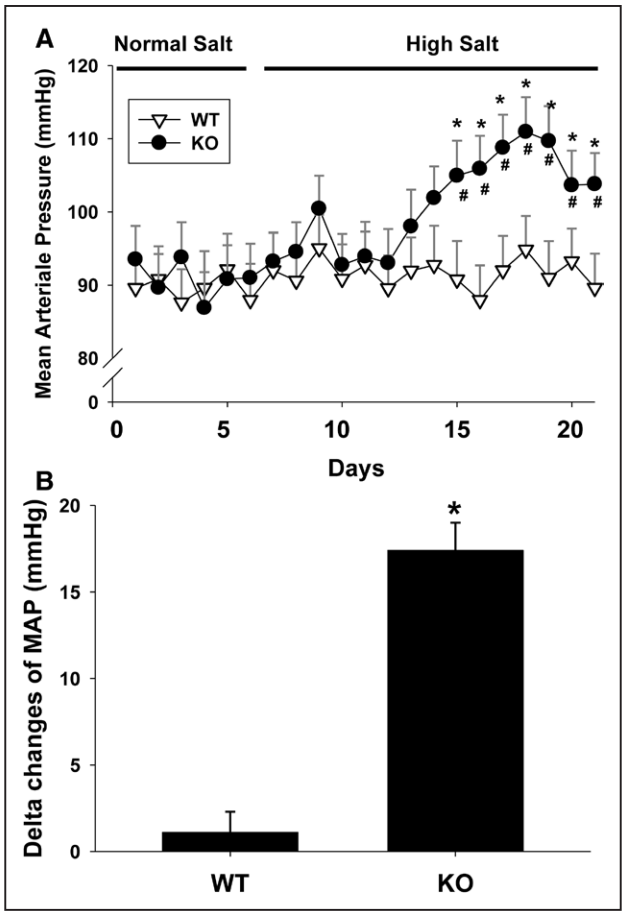

Figure 6. Blood pressure response to a high salt diet. The mice were fed a normal salt diet (0.4 NaCl) for 7 d, then switched to a high salt diet (4% NaCl) for 2 wk. The mean arterial pressure (MAP) was measured over time (**A**) and in delta changes (**B**) in the wild-type (WT; n=8) and the knock-out (KO; n=9) mice (*P<0.01 vs normal salt, #P<0.01 vs WT).

WT; Figure 6). Data about systolic and diastolic blood pressure and heart rate are presented in the online-only Data Supplement.

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 a salt loading. We generated a tissue-specific primary cilia deletion mouse line, in which the primary cilia were deleted from the macula densa and the TAL and demonstrated that the NO generated by the macula densa and TAL was significantly decreased in these 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.

The primary cilia on TALs were easy to identify in the WT mice and were clearly deleted in the KO mice. However, the primary cilia on the macula densa were not as easy to visualize as on the TAL. Because, in most cases, only 1 primary cilium is present on the apical surface of each epithelial cell[7–12] 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.[12] 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 was being produced by NOS1. 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.[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 cilia play an essential role in the NO generation by the TAL. These findings provide a potential mechanism by which primary cilia 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,[41,42] we examined whether primary cilia had any effect on $[Ca^{2+}]_i$. 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 $[Ca^{2+}]_i$ by 60% in the macula densa and by 33% in TAL. The shear stress–induced $[Ca^{2+}]_i$ alterations were blocked in the KO mice, indicating that the primary cilia mediate the shear stress–induced intracellular calcium changes.

We recently found that shear stress stimulates macula densa NOS1 activity mediated by the primary cilia.[12] We previously reported that NOS1β is the primary splice variant and contributes to most of the NO generation by the macula densa,[20,30,44] which inhibits the TGF response.[16–19] 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

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 compensatory 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 demonstrated in this study that deletion of the primary cilia from the TAL decreased NO generation by the TAL. It has been well established that the NO produced by TAL inhibits sodium reabsorption by inhibition of NKCC2 and sodium-hydrogen exchanger cotransporter activities in the TAL, which promotes sodium excretion.[45–48] 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.[49] 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 exhibited 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 primary cilia on the macula densa and TAL play an important role in the control of sodium excretion and hemodynamics.

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 $[Ca^{2+}]_i$, which blunts TGF responsiveness leading to an increase in GFR, promoting sodium excretion 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

None.

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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.

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.